IDENTIFICATION OF DEC, A RECEPTOR WITH C-TYPE LECTIN DOMAINS, NUCLEIC ACIDS ENCODING DEC, AND USES THEREOF

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FIELD OF THE INVENTION

The present invention relates to the identification and characterization of a receptor associated with antigen presentation in immune responses, endocytosis, and trans-epithelial transport. Identification of the receptor, its characterization as having ten lectin-binding domains, and evidence of its role in the uptake and processing of oligosaccharides and oligosaccharide-decorated molecules, *e.g.*, glycoproteins, has important ramifications for modifying immune response, and for trans-epithelial transport of molecules.

BACKGROUND OF THE INVENTION

Dendritic cells are a unique class of leukocytes whose primary function is to capture, process, and present antigens to T cells (Steinman, 1991, Annu. Rev. Immunol. 9:271-96). Interaction between dendritic cells and specific T cells in the peripheral immune system leads to the induction of immune responses, whereas in the thymus presentation by dendritic cells leads to negative selection (Tanaka et al., 1993, Eur. J. Immunol. 23:2614-2621; Matzinger et al., 1989, Nature 338:74-76). Like dendritic cells, thymic epithelial cells present MHC-bound peptides to T cells, but instead of inducing T cell activation or negative selection, thymic epithelial cells direct positive selection (Hugo et al., 1993, Immunol. Rev. 135:133-35; Elliott, Immunol. Rev. 135:215-25). Consistent with the known requirements for interactions with T cells, both dendritic cells and thymic epithelial cells express a number of cell surface proteins that facilitate cell-cell contact and mediate T cell activation (Steinman, 1991, Annu. Rev Immunol. 9:271-96; Hugo et al., 1993, Immunol Rev. 135:133-35; Elliott, Immunol Rev.

135:215-25). An additional fundamental requirement for both dendritic cells and thymic epithelial cells is the uptake and processing of antigen, yet neither cell type is known to express receptors that are specialized for antigen capture or presentation.

Over the decade since its initial isolation by Kraal et al. (J. Exp. Med. 163:981), the monoclonal antibody NLDC-145 has been utilized as a histochemical and flow cytometric marker for mouse dendritic cells (DCs) in a variety of tissues (Kraal et al., *supra*; Crowley et al., 1989, Cell Immunol. 118:108; Vremec et al., 1992, J. Exp. Med. 176:47; Pollard and Lipscomb, 1990, J. Exp. Med. 172:159; Soesatyo et al., 1990, Cell Tiss. Res. 259:587; Austyn et al., 1994, J. Immunol. 152:2401; Lu et al., 1994, J. Exp. Med. 179:1823; and Breel et al., 1988, Immunol. 63:657). The antigen bound by NLDC-145 is also abundant on thymic cortical epithelium. However, cloning and characterization of the NLDC-145 antigen has proved elusive. For one thing, dendritic cell cDNA libraries have not been readily prepared. Dendritic cells themselves are rare, making their RNA extremely rare. Moreover, monoclonal antibodies are not usually effective reagents for screening expression libraries, *e.g.*, a λgt-11 expression library.

Accordingly, there is a need in the art to clone and characterize the antigen recognized by monoclonal antibody NLDC-145.

There is a further need in the art to harness the immunomodulatory abilities of dendritic cells, *e.g.*, to induce tolerance or immunity.

The citation of any reference herein should not be construed as an admission that such reference is prior art to the instant invention.

SUMMARY OF THE INVENTION

The present invention relates to an integral membrane protein, termed herein "DEC," found primarily on dendritic cells, but also found on B cells, brain capillaries, bone marrow stroma, epithelia of intestinal villi and pulmonary airways, as well as cortical epithelium of the thymus and the dendritic cells in the T cell areas of peripheral lymphoid organs. In addition, trace amounts of this protein are found in organs like the liver, heart, and kidney. The murine and human counterparts of DEC have an apparent molecular mass of 205 kDa, the murine counterpart has an isoelectric point at pH 7.5, and carbohydrates comprise about 7 kDa of the total mass of murine DEC. The carbohydrates appear to consist of eight distinct but related biantennary N-linked glycans, with no O-linked glycans present. Because the protein has been found predominantly on Dendritic cells and thymic Epithelial Cells, and has a molecular weight of 205 kDa, it is termed DEC-205. In a specific embodiment, the invention relates to isolation and cloning of human DEC, which is further characterized by having a carboxyl-terminal sequence RHRLHLAGFSSVRYAQGVNEDEIMLPSFHD (SEQ ID NO: 1), and characterized by binding to a rabbit polyclonal antibody raised against full length murine DEC-205, but not reacting with monoclonal antibody NLDC-145. Another characteristic of DEC, based on the deduced amino acid sequence information obtained from cloning the *dec* cDNA is a unique coated pit localization consensus domain or motif on the cytoplasmic tail of the protein, which, as shown in Figure 7C, has regions of homology and regions of dissimilarity between the two counterpart proteins. It has been further discovered that DEC-205 is rapidly internalized via coated vesicles, and delivers bound substances to a multivesicular endosomal compartment that resembles the MHC class-II containing vesicles implicated in antigen processing.

Accordingly, in its primary aspect, the present invention is directed to identification of additional ligands of the DEC-205 receptor, which can be advantageously targeted to dendritic cells and other cells that bear DEC-205.

Targeting antigens for presentation by dendritic cells can provide for tolerance when the dendritic cells are quiescent, or for immune stimulation (*i.e.*, vaccination) when the dendritic cells are activated, *e.g.*, by stimulation with a cytokine or lymphokine, such as colony stimulating factor (CSF).

Furthermore, the presence of DEC on epithelial cells suggests an important role in trans-epithelial transport of molecules, *e.g.*, from the basolateral surface of the lung respiratory epithelium into lung airways or the basolateral surface of the intestinal epithelium into the lumen of the intestines, and from the apical (lumenal) surface of these epithelial to the pulmonary or intestinal circulation, respectively. Thus, the invention provides for parenteral delivery of pharmacological agents, *e.g.*, antibiotics, to infections of the lung or the intestines, by targeting the pharmacological agent with a ligand to DEC, and for systemic delivery of pharmacological agents by aerosolization and inhalation via the lungs, or ingestion and absorption via the intestines.

In a further aspect, the invention provides for targeting pharmacological agents to cross the blood brain barrier via DEC located on the brain capillaries.

Thus, in a preferred aspect, the invention provides a method for identifying a ligand for DEC, comprising contacting a protein comprising at least one DEC lectin domain with a candidate ligand; and detecting binding of the candidate ligand with the DEC lectin domain. Binding of the candidate ligand and the DEC lectin domain indicates that the ligand candidate is a ligand for DEC. In a preferred aspect, the ligand is a saccharide, which binds to one or more of the lectin domains on DEC.

According to one aspect of the invention, the protein comprising at least one DEC lectin domain is expressed by cells as an integral membrane protein, and the candidate ligand is labeled, such that binding of the candidate ligand with the DEC lectin domain is detected by detecting association of the label with the cells. In

another embodiment, the protein comprising at least one DEC lectin domain is solubilized, and the candidate ligand is irreversibly associated with a solid phase support, such that binding of the candidate ligand with the DEC lectin domain is detected by detecting binding of the protein with the solid phase support. In yet another embodiment, the protein comprising at least one DEC lectin domain is irreversibly associated with a solid phase support, and the candidate ligand is labeled, such that binding of the candidate ligand with the DEC lectin domain is detected by detecting association of label with the solid phase support. In one embodiment, the protein comprising at least one DEC lectin domain is a truncated DEC protein; in another embodiment, the protein comprising at least one DEC lectin domain is a full length DEC protein.

The present invention advantageously provides a nucleic acid encoding at least a portion of a DEC protein. Thus, the invention provides for expression of DEC proteins, or truncated fragments thereof, including chimeric proteins, which can be used for identifying a DEC ligand. Moreover, the nucleic acid of the invention comprises at least fifteen base pairs, thus, the nucleic acids of the invention provide useful probes for detecting expression of mRNA for DEC, PCR primers for reverse transcriptase polymerase chain reaction (RT-PCR) amplification of RNA, or for cloning DEC, and probes for the presence of DEC cDNA or genomic DNA, *e.g.*, in a library or cell. In a preferred embodiment, the nucleic acid encodes a human DEC protein. In a specific embodiment, *infra*, a nucleic acid encoding murine DEC is provided.

The present invention further provides an expression vector comprising the nucleic acid encoding DEC, wherein the nucleic acid is a DNA molecule encoding at least a lectin domain of DEC, operatively associated with an expression control sequence. In a further aspect, the invention provides a recombinant host cell comprising the expression vector. In various embodiments, the host cell is a mammalian cell selected from the group consisting of a Chinese hamster ovary

cell, an African Green Monkey COS cell, a Madin-Darby canine kidney cell, and an NIH-3T3 fibroblast cell.

The invention further provides an antibody reactive with a human DEC-205 protein, in particular a monoclonal antibody and a polyclonal antibody.

As mentioned above, the present invention advantageously provides for identifying ligands of DEC, which ligands are capable of targeting a molecule to which they are attached, *i.e.*, conjugated, to a cell bearing DEC *in vitro* or *in vivo*. The ability to target cells that express DEC *in vivo* has important implications from the perspective of specifically targeting dendritic cells, epithelial cells, *e.g.*, of the thymus, small intestine, and lung. Thus, the invention is naturally directed to a pharmaceutical composition comprising a molecule targeted to a tissue selected from the group consisting of pulmonary circulation, intestinal circulation, pulmonary airways, lumen of the small intestine, dendritic cells in the skin and T cell areas of lymphoid organs, thymus, and brain, which molecule is conjugated to a DEC-ligand. Preferably, the DEC-ligand is selected from the group consisting of a carbohydrate that binds DEC and an anti-DEC antibody, and a pharmaceutically acceptable carrier. In specific embodiments, the molecule is selected from the group consisting of an anti-cancer drug, an anti-viral drug, an antibiotic, an anti-parasitic drug, and an anti-inflammatory drug.

In another aspect of the invention related to targeting, a recombinant vector for introduction of a gene into cells selected from the group consisting of dendritic cells, thymic epithelial cells, lung epithelial cells, small intestine epithelial cells, and brain capillary cells comprising a DNA vector conjugated to a DEC-ligand is provided, wherein the DEC-ligand is selected from the group consisting of a carbohydrate that binds DEC and an anti-DEC antibody. In specific embodiments, the DNA vector is selected from the group consisting of a viral vector, a liposome vector, and a naked DNA vector.

In yet a further embodiment, grounded on the ability to target a molecule to dendritic cells, the present invention provides a vaccine comprising an antigen from a pathogen conjugated to a DEC-ligand, wherein the DEC-ligand is selected from the group consisting of a carbohydrate that binds DEC and an anti-DEC antibody, and an immune stimulator. Examples of pathogens include, but are not limited to, a virus, a bacterium, a parasite, and a tumor. The immune stimulator may be selected from the group consisting of a cytokine, a lymphokine, and an adjuvant. In particular, the invention advantageously provides for targeting a molecule that is either a poor immunogen, or that is not immunogenic at all, to dendritic cells for efficient processing (as DEC is shown herein to be associated with antigen processing mechanisms of dendritic cells) and presentation to responsive T lymphocytes.

Alternatively, the invention provides a composition to induce immune suppression comprising an autoantigen or an allergen conjugated to a DEC-ligand, wherein the DEC ligand is selected from the group consisting of a carbohydrate that binds DEC and an anti-DEC antibody, with the proviso that the composition lack immune stimulatory agents. By targeting an autoantigen or allergen to dendritic cells without including stimulatory agents, *e.g.*, cytokines, lymphokines, or adjuvants, the quiescent dendritic cells can process and present antigen. Presentation of antigen by quiescent dendritic cells is believed to induce antigen-specific T cell anergy or immune tolerance. The autoantigen may be selected from the group consisting of myelin basic protein, collagen or a fragment thereof, DNA, a nuclear protein, a nucleolar protein, a mitochondrial protein, and a pancreatic β-cell protein.

It is a primary object of the instant invention to provide ligands for DEC.

Accordingly, an important corolly object of the invention is to identify ligands that specifically bind DEC.

It is a further object to provide nucleic acids encoding DEC.

A related object is to express nucleic acids encoding DEC, or a portion thereof comprising a carbohydrate binding portion of a DEC lectin domain.

These and other objects of the present invention can be readily appreciated by reference to the following Drawings, Detailed Description of the Invention, and Examples.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. The apparent mass of the antigen bound by NLDC-145 is 205 kDa. (A) Immunoprecipitation of (^{35}S)methionine-cysteine-labeled bone marrow DC extracts with immobilized NLDC-145 (*right*) reveals an actively synthesized antigen with an apparent mass > 200 kDa. This antigen is not precipitated by immobilized nonspecific rat IgG2a (*left*). (B) NLDC-145 binds an antigen of 205 kDa in non-reducing Western blots of crude thymic detergent extract. Three-fold serial dilutions of extract, starting at 0.17 thymic equivalents per lane, were loaded onto duplicate gels, in the absence (*left filter*) or presence (*right strip*) of 5% (v/v) 2-mercaptoethanol, and blotted to nitrocellulose. Filters were probed with 10 μg/ml of NLDC-145 IgG, then peroxidase-conjugated anti-rat IgG. Staining patterns were visualized by ECL. No bands were observed on the reducing gel. STD: positions of prestained broad-range molecular mass markers (Bio-Rad) traced from the original filter onto the developed film.

FIGURE 2. Summary: purification of DEC-205 from thymi. All steps were performed at 0-4°C. Leupeptin and PMSF were added to ice-cold buffers just before use.

FIGURE 3. Analysis of purified DEC-205 and step yields during the purification. (A) Reducing 8% acrylamide SDS-PAGE analysis of 5 μg of

purified protein, stained first with Coomassie Brilliant Blue R-250 (left), then counterstained with silver (right). (B) Isovolumic Western blot of key fractions from the purification, stained with 10 μg/ml of NLDC-145 IgG. Fractions in lanes 1-4 and 6-8 were diluted to the volume of the post-nuclear supernatant, so that the intensities of their 205 kDa bands could be compared and yields estimated. Lane 10: intentional five-fold increase in antigen concentration, to demonstrate a "ladder" of minor mAb-reactive bands ranging down to about 80 kDa in apparent mass. Abbreviations: nuc, nuclear; memb, membrane; extr, 0.5% NP-40 extract; nonads, individual nonadsorbed fractions from early (nonads-5) and late (nonads-15) in the column loading process.

FIGURE 4. DEC-205 is an integral membrane protein with a pI of 7.5. (A) Immunoblot of thymic membrane proteins solubilized with detergent, 1 M KCl or 100 mM Na_2CO_3, pH 11.5 (*lanes 1, 2, 3*), and proteins initially insoluble in the high salt and high-pH buffers, but then released from membranes with detergent (*lanes 4 and 5*). The filter was stained with 10 μg/ml of NLDC-145 IgG. (B) Isoelectric focusing of 10 μg of purified DEC-205 under denaturing conditions. A single lane from a silver-stained slab gel is shown, with pH values assigned after elution of ampholytes from a neighboring unstained lane.

FIGURE 5. Studies of the carbohydrates bound to DEC-205. (*A*) DEC-205 is a glycoprotein. Purified 205 kDa protein, transferrin (Tf, positive control), and creatinase (cre, negative control) were electroblotted to nitrocellulose, and the filter was oxidized with $NaIO_4$, converting *vic*-diols within sugars to immobilized aldehydes. Reaction with a digoxigenin (DIG)-labeled hydrazide, followed by an anti-DIG antibody conjugated to alkaline phosphatase, revealed the positions of glycoproteins on the blot. Like transferrin, but unlike creatinase, DEC-205 stains for sugar. (*B*) The glycans on DEC-205 comprise about 7 kDa of its apparent molecular mass. Apotransferrin (aTf) and DEC-205 were either treated (+) or not (-) with anhydrous trifluoromethanesulfonic acid (TFMSA), to nonselectively hydrolyze protein-bound carbohydrates. Both treated proteins exhibited increased

electrophoretic mobility, corresponding to a 5 kDa loss of apparent mass by apotransferrin, and a 7 kDa loss by DEC-205. (*C*) FACE analysis of N-linked glycans released from DEC-205 with PNGase F. Eight bands are resolved, migrating between 5.1 and 10.1 glucose units. $(Glc)_5$, $(Glc)_{10}$: positions of selected bands in a standard oligo-glucose ladder. (D) Exoglycosidase digestions and FACE analysis of the mixture of N-linked glycans released from DEC-205. Lane 1: Undigested N-linked oligosaccharides (the dark band at $(Glc)_3$ in lanes 1-5 is a detergent artifact). Lane 2: digested with α-galactosidase. Lane 3: digested with α-galactosidase plus NANase III. Lane 4: digested with the previous 2 enzymes plus β-galactosidase. Lane 5: digested with the previous 3 enzymes plus β-N-acetylhexoseaminidase. Lane 6: as for lane 5, but 2-fold higher concentration of β-N-acetylhexoseaminidase. Lane 7: digested with the previous 4 enzymes plus α-mannosidase. Lane 8: as for lane 7, but 2-fold higher concentration of α-mannosidase plus α-fucosidase. Lane 9: mannosylchitobiose core standards: FC, fucosylated core; C, non-fucosylated core. (E) Summary of findings from lectin staining and FACE analysis. Two fucosylated core structures are present, with and without bisecting GlcNAc. Further heterogeneity at the termini produces the 8 glycan variants observed in (C).

FIGURE 6. N-terminal amino acid sequence of DEC-205, and blotting by polyclonal antibodies. (A) The amino-terminal sequence (SEQ ID NO: 2), as determined by two different core facilities. A peptide spanning the first 19 residues was synthesized and coupled to KLH for use as an immunogen. (B) Preclearing study: NLDC-145 specifically depletes the 205 kDa bands detected by both polyclonal antibodies. Immunoblots of crude thymic membrane extract, a depleted fraction produced by passing the same extract over the NLDC-145 immunoaffinity column twice, and material eluted from the column. Filters were stained with: 10 μg/ml of NLDC-145 IgG, 0.1 μg/ml of anti-N-terminal peptide IgG, and 0.1 μg/ml of anti-DEC-205 IgG. All three antibodies bind the same protein.

FIGURE 7. Sequence f DEC-205. (A) Schematic representation of DEC-205. (B) The predicted amino acid sequence of DEC-205 (SEQ ID NO:3) is aligned

with the sequences of the bovine PLA2 receptor (SEQ ID NO:4) and the human macrophage mannose receptor (SEQ ID NO:5). Amino acid positions where there is identity among all three proteins are shaded. Protein domains are separated, and consensus amino acids that define C-type CRDs (Weis et al., Science 254:1608-15) are indicated blow the relevant sequence as follows: invariant amino acids are shown in single letter code, θ = aliphatic, χ = aliphatic or aromatic, ϕ = aromatic, Z = E or Q, B = D or N, Ω = D, N, E or Q. The two missing cysteines in CRD 8 are highlighted with a *. Peptide sequences determined by automated Edman degradation from purified DEC-205 protein are overlined and numbered (N ¯ indicates amino terminal, T indicates peptides generated with Trypsin, and L indicates peptides generated with endoproteinase lys-C). (C) Comparison of carboxyl-terminal cytoplasmic domain sequences of human (*top*) (SEQ ID NO:1) and murine (*bottom*) (SEQ ID NO:6) DEC-205. Regions of identity are underlined; regions of similarity are italicized.

FIGURE 8. DEC-205 Expression. Expression of DEC-205 in mouse tissues and transfected Cos-7 cells. A. Northern blot of poly-A+ A extracted from the indicated tissues. Symbols: Br, brain mRNA; DC, dendritic cell mRna; Ht, heart mRNA; Kd, kidney mRNA; Lv, liver mRNA; LN, lymph node mRNA; Sk, skin mRNA; Thm thymus mRNA; tg, tongue mRNA.

FIGURE 9. Endocytosis of DEC-205. Ultrastructural analysis of DEC-205 on dendritic cells with polyclonal rabbit anti-DEC-205 F(ab)'2 fragments and 10nm gold-labeled goat anti-rabbit IgG (Amersham). The bars represent 100 μm. Symbols: MVV, multivesicular endosome; Ly, Lysosome; CP, coated pit; 0', fixation at time zero; 1', fixation after a one-minute incubation at 37°C; 5', fixation after a five-minute incubation at 37°C; 20', fixation after a twenty-minute incubation at 37°C; 60', fixation after a sixty-minute incubation at 37°C.

FIGURE 10. Antigen Presentation. Antigen presentation by Dendritic cells incubated with rabbit anti-DEC-205 antibodies or non-reactive rabbit antibody

controls. IL-2 production by the 2R.50 cells is plotted against the concentration of antibody in the cultures on a log scale. The error bars indicate the standard deviation from the mean. Symbols: anti-DEC-205, cultures that received the indicated amount of rabbit anti-DEC-205 polyclonal IgG; anti-IgG2a, cultures that received the indicated amount of IgG2a specific polyclonal rabbit antibodies; IgG, cultures that received the indicated amount of non-immune rabbit IgG.

FIGURE 11. Selective staining of Langerhans cells with monoclonal and polyclonal antibodies to DEC-205. Cultured epidermal cells were double-labeled with a PE-tagged mAb to class II MHC proteins (y axis) and multiple antibodies to leukocyte antigens, followed by FITC-anti-Ig (x axis). The mean FITC fluorescence intensity for the MHC-II (+) DCs (e.g., *arrows* in *E* and *H*) is shown in the upper right corner of each panel.

(*A-D*) *Specificity*: Langerhans cells stain for DEC-205, but not for macrophage, B cell or T cell antigens. Rat IgG2a hybridoma supernatants were applied.

(*E-X*) *Titrations* of the monoclonal and polyclonal reagents used in subsequent studies:

(*E-G*) Graded doses of NLDC-145 mAb (0.6, 2 and 6 μg/ml).

(*H*) Polyclonal, nonimmune rat IgG2a (6 μg/ml).

(*I-X*) Rabbit IgGs and F(ab')$_2$ fragments at 3, 10, 30 and 100 μg/ml

FIGURE 12. Trypsin sensitivity and resynthesis of DEC-205 epitopes. Langerhans cells (A-F) or lymph node B cells (G-L) that had been cultured overnight were either exposed to 0.25% trypsin for 30 min on ice, or were not treated. The lymph node B cells had been stimulated with LPS to sustain viability. Cells were either stained immediately (d1) or after an additional day of culture (d2). The antibodies were: 30 μg/ml anti-DEC-205 or nonimmune F(ab')$_2$ fragments (A, C, E, G, I, and K); 2 μg/ml NLDC-145 or nonimmune rat IgG2a; or anti-CD45 (clone M1/9, rat IgG2a) hybridoma supernatant (B, D, F, H, J, and L).

FIGURE 13. Expression of DEC-205 by fresh and cultured dendritic cells (arrows) from spleen and skin. Spleen DCs, enriched in the low-density fraction of spleen cells, were identified with anti-CD11c (y axis, A-H) and counterstained with: NLDC-145; $F(ab')_2$ fragments of the anti-DEC-205 polyclonal; and corresponding nonimmune controls. Staining was performed either immediately after flotation (fresh), or after overnight culture. Fresh and cultured Langerhans cells, identified in an epidermal suspension with a mAb to class II MHC proteins (y axis, I-P), are shown for comparison.

FIGURE 14. Expression of class II MHC proteins and DEC-205 by bone marrow DCs grown from progenitors in the presence of GM-CSF. On days 6, 7 and 8 of culture, cells were examined both by flow cytometry (A-F) and on cytocentrifuge slides (G-I) after staining with: 10 μg/ml of TIB-120 (clone M5/114, anti $I\text{-}A^{b,d,q}$, anti $I\text{-}E^{d,k}$) or nonimmune IgG (A, C, E); 30 μg/ml of anti-DEC-205 or nonimmune IgG (B, D, F); or 2 μg/ml of NLDC-145 (G, H, I). (*d6*) At day 6, the cultures contained large proliferating cell aggregates that expressed heterogeneous levels of class II MHC proteins and little DEC-205. The aggregates were dislodged from plastic-adherent stromal cells. (*d7, d8*) Over two subsequent days of culture, the aggregates released large numbers of nonadherent cells with typical dendritic morphology, abundant class II MHC proteins, and high levels of DEC-205.

FIGURE 15. Expression of DEC-205 on peritoneal cells. Peritoneal cells, either resident or in exudates elicited with the indicated proinflammatory agents, were stained with 30 μg/ml of anti-DEC-205 or nonimmune $F(ab')_2$ fragments and FITC-anti-rabbit $F(ab')_2$. The cells were then counterstained with PE-tagged mAbs to macrophages, B cells and T cells. Shown here is staining by PE-anti-Mac-1/CD11b (mAb M1/70, y axis). The Mac-1^{bright} cells are macrophages (*arrowheads*), the Mac-1^{dim} cells are B cells (*arrows*) and the Mac-1 negative cells, T cells. Abbreviations: Con A, concanavalin A; TGC, thioglycollate; BCG, *Mycobacterium bovis* Bacille Calmette-Guérin.

FIGURE 16. Expression of DEC-205 by leukocytes in fresh cell suspensions from three organs. B cells are arrowed. Cells from spleen (A-J), bone marrow (K-T), and peripheral blood (U-δ) were stained with PE-tagged antibodies to subsets of leukocytes (y axis), and counterstained with 30 μg/ml of nonimmune (A-E, K-O, and U-Y) or anti-DEC-205 (F-J, P-T, and Z-δ) $F(ab')_2$ fragments (x axis, FITC). The PE-labeled mAbs reacted with granulocytes (RB6-8C5, anti Gr-1), the integrin CD11b, abundant on granulocytes and macrophages (M1/70, anti-Mac-1), B cells (RA3-6B2, anti-B220/CD45RB), T cells (53-2.1, anti-Thy-1.2/CD90), and class II MHC proteins (AMS-32.1, anti- $I\text{-}A^d$).

FIGURE 17. Immunoblot. Graded doses of whole-cell NP-40 extracts of bone marrow dendritic cells (BMDC), bulk splenic leukocytes (SPL, ca. 65% B cells) and resident peritoneal cells (PC, ca. 70% B cells, 30% macrophages) were transferred to a filter. The filter was stained with 10 μg/ml of NLDC-145 IgG. BMDCs express roughly 10 times more DEC-205 per cell than splenic B cells, and roughly 50 times more than peritoneal B cells.

FIGURE 18. Inability of antibodies to DEC-205 to block dendritic cell stimulatory activity *in vitro*. A mixed leukocyte reaction, where graded doses of mitomycin C-treated spleen dendritic cells were added to 3×10^5 allogeneic lymph node cells in the continuous presence of 10 μg/ml of each of the indicated antibodies, except for anti-DEC-205, which was used at 30 μg/ml. Only the mAb to the costimulator protein B7-2 (clone GL-1) inhibited T cell proliferation. Anti-Igβ: negative control polyclonal to a surface Ig-associated signalling protein on B cells (Sanchez et al., 1993, J. Exp. Med. 178:1049).

FIGURE 20. Expression of DEC-205 in the thymus and in lymph nodes. (*a-c*): Low power of thymus cortex and medulla (M), stained with: monoclonal NLDC-145 (*a*); polyclonal anti-DEC-205 $F(ab')_2$ fragments (*b*); and polyclonal anti-DEC-205 IgG (*c*), all at 10 μg/ml, and counterstained with hematoxylin. Presumptive dendritic cells (*arrowheads*) are scattered throughout the medulla, but

the strongest thymic staining is on cortical epithelium. (*d-f*): Low power views of a mesenteric lymph node, showing a B cell follicle (B), the T cell area of the deep cortex (T), and the medulla (M), stained with: mAb NLDC-145 (*d*), polyclonal anti-DEC-205 $F(ab')_2$ fragments (*e*), and polyclonal anti-DEC-205 IgG (*f*). Darkly-stained dendritic cells are distributed throughout the T cell areas. (*g-i*): Higher power views to show the distribution of DEC-205 at the junction of the thymic cortex and medulla (*g*), within the deep cortex of a lymph node (*h*, a venule is *arrowed*), and in B cell follicles (*i*, no hematoxylin counterstain).

FIGURE 21. Expression of DEC-205 in the spleen. (*a-c*): Low power views of a splenic white pulp nodule, stained with antibodies to: B cells (rabbit anti-Igβ, *a*); DEC-205 (polyclonal anti-DEC-205 IgG, *b*), and class II MHC proteins (mAb M5/114, *c*). The central arteries within the T cell areas are arrowed. The T cell areas contain few B cells (*a*, anti-Igβ), but numerous scattered DEC-205- and class II MHC-positive dendritic cells (*b-c*). B cell follicles are denoted with a "B", and the marginal sinus by arrowheads. (*d-e*): Higher power views of splenic T cell areas (periarterial sheaths, central arteries are arrowed) stained with: mAb NLDC-145 (*d*), polyclonal anti-DEC-205 (*e*), and anti-class II MHC (*f*). Staining for DEC-205 has a punctate quality, in addition to the more prominent staining of dendritic cell bodies.

FIGURE 22. Expression of DEC-205 in several nonlymphoid organs. (*a-d*): Brain capillaries (*arrows*, *a-c*) and small arteries (*arrow*, *d*), stained with: mAb NLDC-145 (*a*), polyclonal anti-DEC-205 $F(ab')_2$ fragments (*b*), and polyclonal anti-DEC-205 IgG (*c-d*). (*e-h*): Lung, showing anti-DEC-205 staining of airway epithelium (*arrows*, *e* and *h*), isolated cells within the lung parenchyma (*arrowheads*, *g* and *h*), and some presumptive alveolar macrophages (*, *h*). Class II MHC proteins (*f*) are not evident within airway epithelium, but there are many positive profiles surrounding the airways (*arrowheads*, *f*). (*i*): An extruded plug of bone marrow. Lacy stromal cells (*arrows*) express low levels of DEC-205. The darker staining of round cells is background staining by peroxidase-expressing

eosinophils. (*j*): Tongue, showing DEC-205 staining of a minority of presumptive Langerhans cells (*arrows*) at suprabasal levels within the oral epithelium, shown as an example of a stratified squamous epithelium. (*k,l*): Jejunum: DEC-205 is expressed by the absorptive epithelial cells of the intestinal villi, with the highest levels observed at the apices of the villi. Numerous cells within the lamina propria also stain darkly, but this staining is again a background of eosinophil peroxidase.

FIGURE 23. Tissue distribution of DEC-205 by immunoblotting. Lysates of the indicated organs were blotted to compare relative levels of expression of DEC-205 protein (*A*, filter stained with mAb NLDC-145) and the LAMP-1 lysosomal membrane antigen (*B*, filter stained with mAb 1D4B). Fifty μg of total protein were loaded in each lane.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an integral membrane protein, termed herein "DEC," found primarily on dendritic cells, but also found on B cells, brain capillaries, bone marrow stroma, epithelia of intestinal villi and pulmonary airways, as well as cortical epithelium of the thymus and the dendritic cells in the T cell areas of peripheral lymphoid organs. In addition, trace amounts of this protein are found in organs like the liver, heart, and kidney. The murine and human counterparts of DEC have an apparent molecular mass of 205 kDa, the murine counterpart has an isoelectric point at pH 7.5, and carbohydrates comprise about 7 kDa of the total mass of murine DEC. The carbohydrates appear to consist of eight distinct but related biantennary N-linked glycans, with no O-linked glycans present. Because the protein has been found predominantly on Dendritic cells and thymic Epithelial Cells, and has a molecular weight of 205 kDa, it is termed DEC-205. Although characterization of the murine and human counterparts of DEC demonstrates the presence of ten carbohydrate binding domains, with a high degree of homology, it is possible that DEC from other species may have more or fewer

such domains. Similarly, DEC may be expressed by other cell types, such as epithelial cells from other tissues or organs.

The invention further relates to cloning of the gene encoding DEC-205, and characterization of the encoded protein. The sequence information indicates that DEC-205 is a receptor with ten C-type lectin domains, which is homologous, or similar, to the macrophage mannose receptor and other related receptors that bind carbohydrates and mediate endocytosis. The human counterpart also appears to have lectin domains. Accordingly, DEC is believed to have a corresponding number of lectin domains, and to be involved in antigen processing by dendritic cells.

Still another aspect of the present invention is the identification of a human DEC protein and gene encoding it.

Another characteristic of DEC, based on the deduced amino acid sequence information obtained from cloning the *dec* cDNA is a unique coated pit localization consensus domain or motif on the cytoplasmic tail of the protein, which, as shown in Figure 7C, has regions of homology and regions of dissimilarity between the two counterpart proteins.

It has been further discovered that DEC-205 is rapidly internalized via coated vesicles, and delivers bound substances to a multivesicular endosomal compartment that resembles the MHC class-II containing vesicles implicated in antigen processing. The invention is also based, in part, on the further discovery that rabbit antibody specific for DEC-205 was efficiently processed by dendritic cells and presented to rabbit-specific T cell clones.

Accordingly, and most importantly, the present invention is directed to identification of additional ligands of the DEC-205 receptor, which can be advantageously targeted to dendritic cells and other cells that bear DEC-205.

Targeting antigens for presentation by dendritic cells can provide for tolerance when the dendritic cells are quiescent, or for immune stimulation (*i.e.*, vaccination) when the dendritic cells are activated, *e.g.*, by stimulation with a cytokine or lymphokine, such as colony stimulating factor (CSF).

Furthermore, the presence of DEC on epithelial cells suggests an important role in trans-epithelial transport of molecules, *e.g.*, from the basolateral surface of the lung respiratory epithelium into lung airways or the basolateral surface of the intestinal epithelium into the lumen of the intestines, and from the apical (lumenal) surface of these epithelial to the pulmonary or intestinal circulation, respectively. Thus, the invention provides for parenteral delivery of pharmacological agents, *e.g.*, antibiotics, to infections of the lung or the intestines, by targeting the pharmacological agent with a ligand to DEC, and for systemic delivery of pharmacological agents by aerosolization and inhalation via the lungs, or ingestion and absorption via the intestines.

In a further aspect, the invention provides for targeting pharmacological agents to cross the blood brain barrier via DEC located on the brain capillaries.

Accordingly, various terms are used throughout this specification, which have the meanings as defined below.

The term "candidate ligand" is used herein to refer to a molecule under consideration of test for its ability to specifically bind to DEC. As discussed in greater detail, *infra*, candidate ligands include, but are by no means limited to, saccharides (*i.e.*, sugars, carbohydrates, or glycans). The term "ligand" as used herein can also refer to an antibody reactive with DEC.

A molecule is "antigenic" when it is capable of specifically interacting with an antigen recognition molecule of the immune system, such as an immunoglobulin (antibody) or T cell antigen receptor. An antigenic polypeptide contains at least

about 5, and preferably at least about 10, amino acids. An antigenic portion of a molecule can be that portion that is immunodominant for antibody or T cell receptor recognition, or it can be a portion used to generate an antibody to the molecule by conjugating the antigenic portion to a carrier molecule for immunization. A molecule that is antigenic need not be itself immunogenic, *i.e.*, capable of eliciting an immune response without a carrier.

A composition comprising "A" (where "A" is a single protein, DNA molecule, vector, recombinant host cell, etc.) is substantially free of "B" (where "B" comprises one or more contaminating proteins, DNA molecules, vectors, etc.) when at least about 75% by weight of the proteins, DNA, vectors (depending on the category of species to which A and B belong) in the composition is "A". Preferably, "A" comprises at least about 90% by weight of the A+B species in the composition, most preferably at least about 99% by weight. It is also preferred that a composition, which is substantially free of contamination, contain only a single molecular weight species having the activity or characteristic of the species of interest.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable

pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, and most preferably prevent, a clinically significant deficit in the activity, function and response of the host. Alternatively, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host.

The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response (Hood et al., *Immunology, Second Ed.*, 1984, Benjamin/Cummings: Menlo Park, California, p. 384). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*. Preferably, the adjuvant is pharmaceutically acceptable.

Various abbreviations used throughout this specification include: PBS, phosphate-buffered saline; mAb, monoclonal antibody; SPF, specific pathogen-free; PMSF, phenylmethylsulfonyl fluoride; DIFP, diisopropyl fluorophosphonate; FACE, fluorophore-assisted carbohydrate electrophoresis.

Genes Encoding DEC, or Fragments, Derivatives, Chimeras, or Analogs Thereof

The present invention contemplates isolation of a gene encoding a functional portion of a DEC receptor of the invention, including a full length, or naturally occurring form of DEC, and any antigenic fragments thereof from any animal, particularly mammalian or avian, and more particularly human, source. As used herein, the term "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids.

Accordingly, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* [B.D. Hames & S.J. Higgins eds. (1985)]; *Transcription And Translation* [B.D. Hames & S.J. Higgins, eds. (1984)]; *Animal Cell Culture* [R.I. Freshney, ed. (1986)]; *Immobilized Cells And Enzymes* [IRL Press, (1986)]; B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-

stranded DNA found, *inter alia*, in linear or circular DNA molecules (*e.g.*, restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5′ to 3′ direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook et al., *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m of 55°, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5x or 6x SCC. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (*see* Sambrook et al., *supra*, 9.50-0.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the

length of the oligonucleotide determines its specificity (*see* Sambrook et al., *supra*, 11.7-11.8). Preferably a minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; more preferably at least about 15 nucleotides; most preferably the length is at least about 20 nucleotides.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (*e.g.*, mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3′ to the coding sequence.

Expression control sequences, e.g., transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, terminators, and the like, that provide for the expression of a coding sequence in a host cell. In eukaryotic cells, polyadenylation signals are control sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control of" or "operatively associated with" a transcriptional and translational control sequence in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced and translated into the protein encoded by the coding sequence.

A "signal sequence" is included at the beginning of the coding sequence of a protein to be expressed on the surface of a cell. This sequence encodes a signal peptide, N-terminal to the mature polypeptide, that directs the host cell to translocate the polypeptide. The term "translocation signal sequence" is used herein to refer to this sort of signal sequence. Translocation signal sequences can be found associated with a variety of proteins native to eukaryotes and prokaryotes, and are often functional in both types of organisms.

As used herein, the term "sequence homology" in all its grammatical forms refers to the relationship between proteins that possess a "common evolutionary origin," including proteins from superfamilies (*e.g.*, the immunoglobulin superfamily) and homologous proteins from different species (*e.g.*, myosin light chain, etc.) (Reeck et al., 1987, Cell 50:667).

Accordingly, the term "sequence similarity" in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that do not share a common evolutionary origin (*see* Reeck et al., *supra*).

Two DNA sequences are "substantially homologous" or "substantially similar" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions

is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

Similarly, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 70% of the amino acids are identical, or functionally identical. Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program.

The term "corresponding to" is used herein to refer similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

A gene encoding DEC, whether genomic DNA or cDNA, can be isolated from any source, particularly from a human cDNA or genomic library. Methods for obtaining DEC gene are well known in the art, as described above (*see, e.g.*, Sambrook et al., 1989, *supra*). In specific embodiment, *infra*, a cDNA encoding murine DEC-205 is isolated from a dendritic cell library. In addition, probes derived from the murine gene were used to isolate the corresponding human *dec* cDNA and the murine genomic *dec* gene.

Accordingly, any animal cell potentially can serve as the nucleic acid source for the molecular cloning of a *dec* gene. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), and preferably is obtained from a cDNA library prepared from tissues with high level expression of the protein (*e.g.*, a dendritic cell cDNA or thymic epithelial cDNA library, since these are the cells that evidence highest levels of expression of DEC), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or

fragments thereof, purified from the desired cell (See, for example, Sambrook et al., 1989, *supra*; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will not contain intron sequences. Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired *dec* gene may be accomplished in a number of ways. For example, if an amount of a portion of a *dec* gene or its specific RNA, or a fragment thereof, is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). For example, a set of oligonucleotides corresponding to the partial amino acid sequence information obtained for the DEC protein can be prepared and used as probes for DNA encoding DEC, as was done in a specific example, *infra*, or as primers for cDNA or mRNA (*e.g.*, in combination with a poly-T primer for RT-PCR). Preferably, a fragment is selected that is highly unique to DEC of the invention. Those DNA fragments with substantial homology to the probe will hybridize. As noted above, the greater the degree of homology, the more stringent hybridization conditions can be used. In a

specific embodiment, the human *dec* cDNA was cloned using a 300 base-pair probe derived from the 3′ coding sequence of murine *dec* cDNA. The human cDNA was obtained from a B lymphoma library, using high stringency hybridization conditions (0.1 SSC, 65°). Thus, high stringency hybridization conditions are favored to identify a homologous *dec* gene from other species.

Further selection can be carried out on the basis of the properties of the gene, *e.g.*, if the gene encodes a protein product having the isoelectric, electrophoretic, amino acid composition, or partial amino acid sequence of DEC protein as disclosed herein. Thus, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, *e.g.*, has similar or identical electrophoretic migration, isoelectric focusing or non-equilibrium pH gel electrophoresis behavior, proteolytic digestion maps, or antigenic properties as known for DEC. For example, the rabbit polyclonal antibody to murine DEC, described in detail *infra*, can be used to confirm expression of DEC, both murine and human counterparts. In another aspect, a protein that has an apparent molecular weight of 205 kDa, and which is specifically digested to form a defined ladder (rather than a smear) of lower molecular weight bands, is a good candidate for DEC.

A *dec* gene of the invention can also be identified by mRNA selection, *i.e.*, by nucleic acid hybridization followed by *in vitro* translation. In this procedure, nucleotide fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified *dec* DNA, or may be synthetic oligonucleotides designed from the partial amino acid sequence information. Immunoprecipitation analysis or functional assays (*e.g.*, tyrosine phosphatase activity) of the *in vitro* translation products of the products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments, that contain the desired sequences. In addition, specific mRNAs may be

selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against DEC, such as the rabbit polyclonal anti-murine DEC antibody described herein.

A radiolabeled *dec* cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA may then be used as a probe to identify homologous *dec* DNA fragments from among other genomic DNA fragments.

The present invention also relates to cloning vectors containing genes encoding analogs and derivatives of DEC of the invention, that have the same or homologous functional activity as DEC, and homologs thereof from other species. The production and use of derivatives and analogs related to DEC are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type DEC.

In another aspect, a DEC protein of the invention can be prepared by substituting a lectin domain or domains from another protein, such as the mannose receptor of macrophages or the phospholipase receptor on muscle, for those found in DEC 205.

DEC derivatives can be made by altering encoding nucleic acid sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Preferably, derivatives are made that have enhanced or increased functional activity relative to native DEC. Alternatively, such derivatives may encode soluble fragments of DEC extracellular domain that have the same or greater affinity for the natural ligand of DEC of the invention. Such soluble derivatives may be potent inhibitors of ligand binding to DEC.

Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a *dec* gene may be used in

the practice of the present invention. These include but are not limited to allelic genes, homologous genes from other species, and nucleotide sequences comprising all or portions of *dec* genes which are altered by the substitution of different codons that encode the same amino acid residue within the sequence, thus producing a silent change. Likewise, the DEC derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a DEC protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a conservative amino acid substitution. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity, which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point.

The genes encoding DEC derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned DEC gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1989, *supra*). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of DEC, care should be taken to ensure that the modified gene remains within the same translational reading frame as the DEC gene, uninterrupted

by translational stop signals, in the gene region where the desired activity is encoded.

Additionally, the DEC-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Preferably, such mutations enhance the functional activity of the mutated DEC gene product. Alternatively, deletion mutants can be produced that encode fragments of DEC, *e.g.*, one or a few of the lectin domains (*see* Taylor et al., 1992, J. Biol. Chem. 267:1719). Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem. 253:6551; Zoller and Smith, 1984, DNA 3:479-488; Oliphant et al., 1986, Gene 44:177; Hutchinson et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:710), use of TAB® linkers (Pharmacia), etc. PCR techniques are preferred for site directed mutagenesis (see Higuchi, 1989, "Using PCR to Engineer DNA", in *PCR Technology: Principles and Applications for DNA Amplification*, H. Erlich, ed., Stockton Press, Chapter 6, pp. 61-70).

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Examples of vectors include, but are not limited to, *E. coli*, bacteriophages such as lambda derivatives, or plasmids such as pBR322 derivatives or pUC plasmid derivatives, *e.g.*, pGEX vectors, pmal-c, pFLAG, etc. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini;

these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated. Preferably, the cloned gene is contained on a shuttle vector plasmid, which provides for expansion in a cloning cell, *e.g.*, *E. coli*, and facile purification for subsequent insertion into an appropriate expression cell line, if such is desired. For example, a shuttle vector, which is a vector that can replicate in more than one type of organism, can be prepared for replication in both *E. coli* and *Saccharomyces cerevisiae* by linking sequences from an *E. coli* plasmid with sequences form the yeast 2μ plasmid.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionation, can be done before insertion into the cloning vector.

Expression of DEC Polypeptides

The nucleotide sequence coding for DEC, or antigenic fragment, derivative or analog thereof, or a functionally active derivative, including a chimeric protein, thereof, can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Such elements are termed herein a "promoter." Thus, the nucleic acid encoding DEC of the invention is operationally associated with a promoter in an expression vector of the invention. Both cDNA and genomic sequences can be cloned and expressed under control of such regulatory sequences. An expression vector also preferably includes a replication origin.

The necessary transcriptional and translational signals can be provided on a recombinant expression vector, or they may be supplied by the native gene encoding DEC and/or its flanking regions.

Potential host-vector systems include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

A recombinant DEC protein of the invention, or functional fragment, derivative, chimeric construct, or analog thereof, may be expressed chromosomally, after integration of the coding sequence by recombination. In this regard, any of a number of amplification systems may be used to achieve high levels of stable gene expression (*See* Sambrook et al., 1989, *supra*).

The cell into which the recombinant vector comprising the nucleic acid encoding DEC is cultured in an appropriate cell culture medium under conditions that provide for expression of DEC by the cell.

Any of the methods previously described for the insertion of DNA fragments into a cloning vector may be used to construct expression vectors containing a gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombination (genetic recombination).

Expression of DEC protein may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters which may be used to control DEC gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3′ long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci.

U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and the animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals.

Expression vectors containing a nucleic acid encoding a DEC of the invention can be identified by four general approaches: (a) PCR amplification of the desired plasmid DNA or specific mRNA, (b) nucleic acid hybridization, (c) presence or absence of selection marker gene functions, and (d) expression of inserted sequences. In the first approach, the nucleic acids can be amplified by PCR to provide for detection of the amplified product. In the second approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted marker gene. In the third approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "selection marker" gene functions (*e.g.*, β-galactosidase activity, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. In another example, if the nucleic acid encoding DEC is inserted within the "selection marker" gene sequence of the vector, recombinants containing the DEC insert can be identified by the absence of the DEC gene function. In the fourth approach, recombinant expression vectors can be identified by assaying for the activity, biochemical, or immunological characteristics of the gene product expressed by the recombinant, provided that the expressed protein assumes a functionally active conformation.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, cleavage [*e.g.*, of signal sequence]) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an nonglycosylated core protein product. However, the transmembrane DEC protein expressed in bacteria may not be properly folded. Expression in yeast can produce a glycosylated product, although the pattern of glycosylation will likely differ from that obtained by expression in a mammalian cell. Expression in eukaryotic cells can increase the likelihood of "native" glycosylation and folding of a heterologous protein. Moreover, expression in mammalian cells, such as Chinese hamster ovary (CHO), African Green Monkey COS cells, and fibroblast NIH-3T3 cells (*e.g.*, 293 cells), can provide a tool for reconstituting, or constituting, DEC activity. Furthermore, different vector/host expression systems may affect processing reactions, such as proteolytic cleavages, to a different extent.

In a preferred aspect of the invention, DEC is introduced into model epithelial cells, such as Madin-Darby canine kidney (MDCK) cells, for investigation of the efficacy and rate of trans-epithelial migration of ligands or molecules targeted to

DEC. Alternatively, as set forth below, the *dec* gene can be introduced into epithelial or dendritic cells for gene therapy, either by *in vivo* or *ex vivo* gene transfer.

Vectors are introduced into the desired host cells by methods known in the art, *e.g.*, transfection, electroporation, microinjection, transduction, cell fusion, DEAE (diethyl aminoethyl) dextran, calcium phosphate precipitation, lipofection (lysosome fusion), use of a gene gun, or a DNA vector transporter (see, *e.g.*, Wu et al., 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

A recombinant DEC protein expressed as an integral membrane protein can be isolated and purified by standard methods. Generally, the integral membrane protein can be obtained by lysing the membrane with detergents, such as but not limited to, sodium dodecyl sulfate (SDS), ~~Triton~~ TRITON X-100, ~~Nonidet~~ NONIDET P-40 (NP-40), digoxin, sodium deoxycholate, and the like, including mixtures thereof. Solubilization can be enhanced by sonication of the suspension. In a specific embodiment, *infra*, DEC-205 is solubilized from thymic membrane pellets in a buffer containing 0.5% NP-40. Soluble forms of the protein can be obtained by collecting culture fluid, or solubilizing inclusion bodies, *e.g.*, by treatment with detergent, and if desired sonication or other mechanical processes, as described above. The solubilized or soluble protein can be isolated using various techniques, such as polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, 2-dimensional gel electrophoresis, chromatography (*e.g.*, ion exchange, affinity, immunoaffinity, and sizing column chromatography), centrifugation, differential solubility, immunoprecipitation, or by any other standard technique for the purification of proteins. In a specific embodiment, *infra*, DEC-205 was purified using an affinity column with monoclonal antibody NLDC-145. In a another embodiment, *infra*, DEC-205 was purified by immunoprecipitation with either monoclonal antibody NLDC-145.

Characterization of DEC Structure

Once a recombinant which expresses the DEC gene sequence is identified, and expression of an abundance of the protein is achieved, the recombinant DEC product can be analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labelling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

For example, the ability of the expressed protein, or a fragment comprising the cytoplasmic domain thereof, to mediate endocytosis and targeting to coated pits, and thence to endocytic vesicles associated with Class II MHC processing, can be determined. In one embodiment, *infra*, endocytosis was evaluated by electron microscopy, using an anti-DEC antibody and a gold-labeled secondary antibody reactive with the anti-DEC antibody. In another embodiment, the ability to process and present antigen is evaluated by assaying antibody-specific T cell proliferation in response to processing of anti-DEC antibody and a control non-specific antibody.

The structure of DEC of the invention can be analyzed by various methods known in the art. Preferably, the structure of the various domains, particularly the lectin binding and cytoplasmic domains, is analyzed. Structural analysis can be performed by identifying sequence similarity with other known proteins. The degree of similarity (or homology) can provide a basis for predicting structure and function of DEC, or a domain thereof. In a specific embodiment, sequence comparisons can be performed with sequences found in GenBank, using, for example, the FASTA and FASTP programs (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85:2444-48).

The protein sequence can be further characterized by a hydrophilicity analysis (*e.g.*, Hopp and Woods, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the DEC protein.

Secondary structural analysis (*e.g.*, Chou and Fasman, 1974, Biochemistry 13:222) can also be done, to identify regions of DEC that assume specific secondary structures.

Manipulation, translation, and secondary structure prediction, as well as open reading frame prediction and plotting, can also be accomplished using computer software programs available in the art.

By providing an abundant source of recombinant DEC, the present invention enables quantitative structural determination of DEC, or domains thereof. In particular, enough material is provided for nuclear magnetic resonance (NMR), infrared (IR), Raman, and ultraviolet (UV), especially circular dichroism (CD), spectroscopic analysis. In particular NMR provides very powerful structural analysis of molecules in solution, which more closely approximates their native environment (Marion et al., 1983, Biochem. Biophys. Res. Comm. 113:967-974; Bar et al., 1985, J. Magn. Reson. 65:355-360; Kimura et al., 1980, Proc. Natl. Acad. Sci. U.S.A. 77:1681-1685). Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstom, A., 1974, Biochem. Exp. Biol. 11:7-13).

More preferably, co-crystals of DEC and a DEC-specific ligand can be studied. Analysis of co-crystals provides detailed information about binding, which in turn allows for rational design of ligand agonists and antagonists. Computer modeling can also be used, especially in connection with NMR or X-ray methods (Fletterick, R. and Zoller, M. (eds.), 1986, Computer Graphics and Molecular Modeling, in *Current Communications in Molecular Biology*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

In yet a further embodiment, a putative DEC of the invention can be tested to determine whether it cross-reacts with an antibody specific for murine DEC-205. For example, the putative DEC can be reacted with a rabbit polyclonal antibody, as

described in the Example, *infra*, to determine whether it binds. Alternatively, a DEC protein can be used to generate antibodies, which can be tested for cross reactivity with DEC-205 from mice or human sources. The degree of cross reactivity provides information about structural homology or similarity of proteins.

The carbohydrate composition of DEC can be studied by various means known in the art, including but not limited to, lectin binding, chemical analysis, immunoassay, immunochemical analysis (*e.g.*, by converting glycoconjugates to digoxigenin-labeled hydrazones after periodate oxidation of *vic*-diols), chemical deglycosylation, enzymatic deglycosylation, and exoglycosidase digestions followed by FACE (fluorophore-assisted carbohydrate electrophoresis) analysis.

Ligands for DEC

Most importantly, the present invention advantageously provides for identifying ligands of DEC, *e.g.*, carbohydrate ligands that bind to one or more of the lectin domains of DEC. Such ligands are especially useful for targeting binding to DEC. As used herein, "ligand" has its ordinary meaning, *i.e.*, a molecule capable of specifically binding to a receptor, in this case DEC. As used herein, the term "carbohydrate ligand" refers to a carbohydrate or sugar that is capable of specifically binding DEC. Generally, such carbohydrates, alternatively termed herein "glycans," "saccharides," or "oligosaccharides," are the carbohydrate portion of a glycoprotein.

Identification and isolation of a gene encoding DEC of the invention provides for expression of the receptor, or truncated portions thereof, in quantities greater than can be isolated from natural sources, in recombinant cells for classical receptor binding experiments, or in indicator cells that are specially engineered to indicate the activity of a receptor expressed after transfection or transformation of the cells. According, the present invention contemplates identifying specific ligands DEC using various screening assays known in the art. The recombinantly expressed protein can comprise one or more DEC lectin domains, and may be a truncated

form of the native protein or portions of the native protien expressed as a chimeric construct with another protein.

Any screening technique known in the art can be used to screen for DEC ligands. The present invention contemplates screens for small molecule ligands or ligand analogs and mimics, as well as screens for natural ligands that bind to DEC *in vivo*. In particular, the present invention provides for identification of carbohydrate groups that bind DEC, and more specifically, identification of carbohydrate groups that bind DEC with high affinity and specificity.

In a preferred aspect of the invention, detection of DEC ligands is accomplished by binding solubilized DEC or DEC fragments to columns prepared from sugars or glycans conjugated to a solid phase support, such as SEPHAROSE (Taylor et al., 1992, J. Biol. Chem. 267:1719). In particular, the invention contemplates dissecting the ligand specificity of various of the lectin domains by expressing truncated mutant DEC proteins comprising only one or a few of the domains. Alternatively, candidate glycans can be conjugated to a carrier protein, such as bovine serum albumin, which is labelled, *e.g.*, with ^{125}I, and binding detected to DEC or DEC fragments expressed by a cell, such as a recombinant cell as described *supra* (Taylor et al., *supra*). In yet another embodiment, binding of labeled glycan-carrier protein is evaluated in microtiter assays, as described (Taylor and Drickamer, 1993, J. Biol. Chem. 268:399). Candidate carbohydrate ligands include, but are not limited to, mannose, fucose, N-acetyl-glucosamine, glucose, galactose, N-acetyl-galactosamine, to mention but a few such carbohydrates. Other ligand candidates include disaccharides, and larger order polysaccharides, *e.g.*, such as are recognized by various lectins.

As used herein, the term "detection of binding" refers to any of the miriad techniques commonly employed to detect the association of one molecule with another, *i.e.*, DEC-ligand with DEC. These techniques include the immunoassay techniques discussed *infra*, or modifications thereof, and generally depend on

detecting association of a label conjugated with one of the binding entities, either the DEC-lectin containing polypeptide or the candidate ligand, with the other entity, which may be found on a solid phase support or a cell. However, detection of binding can be accomplished indirectly, by detecting the absence of a labeled binding entity, *e.g.*, from supernatant. In a further aspect, binding can be detected by first removing unbound substances, followed by removing the labeled entity (*e.g.*, using a chaotropic agent) from the bound pair. These and other techniques for detecting binding of one entity to another are well known in the art.

For solid phase or heterogeneous phase assays, one entity of the binding pair will be irreversibly associated with a solid phase support, such as a bead (*e.g.*, SEPHAROSE), latex particle, chromatographic support, magnetic particle, silica particle, silicon wafer, or a plastic microtiter plate. The term "irreversibly associated" refers to covalent or non-covalent binding, characterized by no dissociation, or a rate of dissociation that is so low in comparison to the assay time that it is virtually undetectable.

Knowledge of the primary sequence of DEC, and the similarity of that sequence with proteins of known function, can provide an initial clue as the inhibitors or antagonists of the protein. In the present instance, correlation of the deduced sequence of DEC with the sequences of mannose receptor of macrophage and phospholipase receptor on muscle, assisted characterization of DEC as a receptor with multiple lectin domains. Identification and screening of antagonists is further facilitated by determining structural features of the protein, *e.g.*, using X-ray crystallography, neutron diffraction, nuclear magnetic resonance spectrometry, and other techniques for structure determination, as described above.

In a specific embodiment, identification of carbohydrate ligands for DEC will be accomplished by attaching known glycans to a protein such as the classic neo-glycoprotein, bovine serum albumin, or ovalbumin, or creatinase. In particular, it is advantageous to use a protein that is not naturally glycosylated, so that only the

effects of an added glycan are being assayed. The binding assay may comprise a classical binding assay, as described above, or may involve an antigen processing assay, by evaluating stimulation of antigen-specific T lymphocytes. In this regard, a large number of T cell lines and clones specific for BSA and ovalbumin are available: the ability of neo-glycosylated BSA or ovalbumin to efficiently stimulate specific T cell proliferation is indicative of the ability of the glycan conjugated to the BSA or ovalbumin to bind to DEC.

In another embodiment, the heavily glycosylated protein fetuin, present in fetal calf serum, can be used to evaluate glycan ligands. A fetuin binding system, based on T cell activation or endocytosis of a marker, can be developed. Specific glycosidases can be used to specifically "knock-out" glycans, and the ability of the modified fetuin to function in the binding system evaluated. Diminishment of functional activity would indicate that the enzymatically modified sugar residue was involved in binding to DEC.

In a further aspect, the observation that the ninth and tenth lectin domains of DEC may be involved in membrane associated antibody-mediated antigen presentation, by "chaperoning" antibody into endosomes, suggests that these domains are specific for binding carbohydrates found on cell surface immunoglobulin molecules.

Another approach uses recombinant bacteriophage to produce large libraries. Using the "phage method" (Scott and Smith, 1990, Science 249:386-390; Cwirla, et al., 1990, Proc. Natl. Acad. Sci., 87:6378-6382; Devlin et al., 1990, Science, 249:404-406), very large libraries can be constructed (10^6-10^8 chemical entities). A second approach uses primarily chemical methods, of which the Geysen method (Geysen et al., 1986, Molecular Immunology 23:709-715; Geysen et al. 1987, J. Immunologic Method 102:259-274) and the recent method of Fodor et al. (1991, Science 251, 767-773) are examples. Furka et al. (1988, 14th International Congress of Biochemistry, Volume 5, Abstract FR:013; Furka, 1991, Int. J. Peptide Protein Res. 37:487-493), Houghton (U.S. Patent No. 4,631,211, issued December 1986) and

Rutter et al. (U.S. Patent No. 5,010,175, issued April 23, 1991) describe methods to produce a mixture of peptides that can be tested as agonists or antagonists.

In another aspect, synthetic libraries (Needels et al., 1993, "Generation and screening of an oligonucleotide encoded synthetic peptide library," Proc. Natl. Acad. Sci. USA 90:10700-4; Lam et al., International Patent Publication No. WO 92/00252 and U.S. Patent No. 5,382,513, issued January 17, 1995, each of which is incorporated herein by reference in its entirety), and the like can be used to screen for ligands according to the present invention.

Alternatively, assays for binding of soluble ligand to cells that express recombinant forms of a ligand binding domain or domains (preferably domains) of DEC can be performed. As discussed in the Examples, *infra*, the presence of multiple lectin domains on DEC may contribute to the affinity and specificity of binding to glycans.

The screening can be performed with recombinant cells that express the DEC, or alternatively, using purified receptor protein, *e.g.*, produced recombinantly, as described above. For example, the ability of labeled, soluble or solubilized DEC that includes the ligand-binding portion of the molecule, to bind ligand can be used to screen libraries, as described in the foregoing references.

Antibodies to DEC

According to the invention, DEC produced recombinantly or by chemical synthesis, and fragments or other derivatives or analogs thereof, including fusion proteins, may be used as an immunogen to generate antibodies that recognize DEC. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, *infra*, a rabbit polyclonal antibody is prepared against the N-terminal amino acid sequence of DEC-205. In another, a polyclonal antibody against intact, purified, DEC-205 was generated.

Various procedures known in the art may be used for the production of polyclonal antibodies to DEC, or derivative or analog thereof. For the production of antibody, various host animals can be immunized by injection with the non-allogeneic DEC, or a derivative (*e.g.*, fragment or fusion protein) thereof, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, the DEC or fragment thereof can be conjugated to an immunogenic carrier, *e.g.*, bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, as described above.

For preparation of monoclonal antibodies directed toward DEC, or fragment, analog, or derivative thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, J. Bacteriol. 159-870; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for an DEC together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce DEC-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for DEC, or its derivatives, or analogs.

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention. For example, to select antibodies which recognize a specific epitope of DEC, one may assay generated hybridomas for a product which binds to a DEC fragment containing such epitope. For selection of an antibody specific to DEC from a particular species of animal, one can select on the basis of positive

binding with DEC expressed by or isolated from cells of that species of animal, and the absence of binding to DEC from other species. Binding to DEC may be detected as binding to dendritic cells that express DEC.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the DEC, *e.g.*, for Western blotting, imaging DEC *in situ*, measuring levels thereof in appropriate physiological samples, etc. The antibodies of the present invention advantageous provide for detecting and enumerating human dendritic cells. Alternatively, such antibodies can be used to isolate human dendritic cells, *e.g.*, by panning. In yet another embodiment, the antibodies of the invention can be used to target molecules to human dendritic cells. It will be recognized that this is a significant advantage, since the prior art antibody of Kraal et al. failed to recognize human DEC.

Antibodies that are targeted to DEC and participate in the activity of DEC, *e.g.*, endocytosis, can be generated. Such antibodies can be tested using the assays described *supra* for identifying ligands. In a specific embodiment, a rabbit polyclonal anti-DEC antibody targets binding of DEC, is endocytosed, and is efficiently presented to immunoglobulin-specific T cells.

Targeting Molecules to DEC

The present invention advantageously provides for targeting molecules to DEC for immune modulation, *e.g.*, stimulation of T cell immunity, suppression immunity or induction of T cell anergy, and clonal deletion mechanism; trans-epithelial transport, with delivery of a molecule across epithelium into the pulmonary circulation or intestinal circulation, or from the bloodstream into the pulmonary or intestinal lumen; and crossing the blood brain barrier. In particular, a ligand for DEC, as described *supra*, or an antibody reactive with DEC (or a DEC-binding portion thereof), as described *supra*, is conjugated to a molecule which is to be targeted to DEC.

Immunomodulation

With respect to immunomodulation, the present invention provides for both stimulating T cell-mediated immune responses, particularly for vaccination, and inducing tolerance, particularly with respect to autoimmunity.

Stimulation of T cell immunity can be effected by introducing an antigen, *e.g.*, ma weak or poorly immunogenic antigen, conjugated to a DEC-binding moiety (ligand or antibody) into a subject, along with a factor that activates the dendritic cells that initially present antigen to the T cells. Dendritic cell activation can be accomplished by use of an adjuvant, such as an adjuvant as described above, which has the ability to induce a generalized immune response. Alternatively, the "vaccine" of the invention may comprise the antigen conjugated to the DEC-binding moiety and a cytokine or a lymphokine, such as granulocyte-macrophage colony stimulating factor (GM-CSF), or some other CSF. Suitable antigens for use in such a vaccine include bacterial, viral, parasite, and tumor antigens.

Alternatively, the present invention provides for inducing tolerance. Tolerance is desirable to avoid detrimental immune responses, in particular, autoimmunity and allograft rejection. Presentation of antigen by non-activated dendritic cells, *e.g.*, in the skin and T cell areas of the lymphoid organs, induces T cell anergy, and possibly causes destruction of the responder clone. Thus, in one embodiment, tolerance is induced by administering an antigen modified by conjugation with a DEC-binding moiety under conditions that promote dendritic cell quiescence, *e.g.*, in the absence of an infection, without adjuvant, using pyrogen-free pharmaceutical carriers, and in the absence of additional lymphokines or cytokines.

It is further believed that high level expression of DEC may act as a tolerizing influence. Accordingly, the invention further relates to introducing recombinant dendritic cells, or cell recombinantly modified to express both DEC and MHC Class II, into a subject, along with antigen conjugated to a DEC-binding moiety.

Alternatively, the *dec* gene can be targeted to appropriate cells *in vivo*, for gene therapy.

In a further embodiment, tolerance can be induced through the clonal deletion mechanism. In particular, antigen conjugated with a DEC-binding moiety can be introduce into a subject, preferably directly into the thymus, either by targeting or physical injection, for processing and presentation by the thymic epithelium and medullary dendritic cells. This processing and presentation step is believed to be involved in the selection process to eliminate autoreactive T cells, *i.e.*, clonal deletion. In a further aspect, the level of expression of DEC may be manipulated, *e.g.*, by introducing additional *dec* genes into the thymic epithelium and medullary dendritic cells.

Attractive candidates for conjugation with a DEC-ligand to induce tolerance, T cell anergy, or clonal deletion include, but by no means are limited to, allergenic substances, autoantigens such as myelin basic protein, collagen or fragments thereof, DNA, nuclear and nucleolar proteins, mitochondrial proteins, pancreatic β-cell proteins, and the like (*see* Schwarz, 1993, In *Fundamental Immunology, Third Edition*, W.E. Paul (Ed.), Raven Press, Ltd.: New York, pp. 1033-1097).

Trans-Epithelial Migration

In another embodiment, a molecule can be targeted for trans-epithelial migration by conjugating it with a DEC-binding moiety. In one aspect of the invention, the invention provides for targeting a therapeutic molecule for absorption across lung or intestinal epithelium. Thus, the invention provides for delivering an aerosolized therapeutic agent by inhalation, *i.e.*, by pulmonary administration of the drug. In another aspect, the invention provides for delivery of a therapeutic agent by DEC-mediated absorption across the small intestine. In particular, the invention advantageously provides for absorption, or more accurately, trans-mucosal migration, of hydrophilic molecules, which are usually not as easily absorbed as

hydrophobic molecules. This aspect of the invention takes advantage of the presence of DEC on the apical (or lumenal) surface of the epithelial cells.

In another aspect of the invention, the presence of DEC on the basolateral surface of the epithelial cells provides a route for transport of a molecule conjugated to a DEC-ligand from the bloodstream into the lumen of the lung or the small intestine. This delivery route can be very important for administration of an acid labile, hydrophilic therapeutic agent to the intestines. Such a drug cannot be ingested, as the acid conditions present in the stomach would result in its destruction; transport of such a drug from the bloodstream to the lumen of the intestines would not readily occur spontaneously, since a hydrophilic agent does not have a significant partition coefficient across cell membranes. In specific embodiments, the present invention provides for administration of chemotherapeutic agents and antibiotics, particularly anti-parasite drugs, by conjugating them to a ligand for DEC, administering the agent parenterally, preferably intravenously, such that the drug is targeted for transport from the basolateral surface of the intestinal epithelium to the lumenal surface.

In the same way, a therapeutic agent may be targeted for delivery from the bloodstream to the airways of the lung by targeting the DEC receptor on the basolateral surface of the lung epithelium. Such a delivery system would be particularly advantageous for delivery of drugs to individuals with impaired lung capacity, *e.g.*, who cannot inhale adequately, and thus, for whom administration via the bloodstream is indicated. Such lung impairments include, but are not limited to, pneumonia, emphysema, lung cancer, adult respirator distress syndrome, dyspnea, hemoptysis, chronic obstructive pulmonary disease (COPD), fibrogenic dust diseases, pulmonary fibrosis, organic dust diseases, chemical injury, smoke injury, thermal injury (burn or freeze), asthma (allergy, bronchoconstriction, other causes of asthma, e.g., irritants), hypersensitivity pneumonitis, Goodpasture's Syndrome, pulmonary vasculitis, and immune complex-associated inflammation. Thus, the invention provides for administration of antibiotics, anti-inflammatory

agents, complement inhibitors (*e.g.*, complement receptor 1 [CD35]), and the like for trans-epithelial migration into the lumen of the lung.

Trans-Blood Brain Barrier Migration

In still another embodiment, a molecule targeted for the brain can be conjugated to a DEC-binding moiety. The molecule would then bind to DEC found in the capillaries of the brain, which are believed to promote trans-blood brain barrier transport or migration. Presently, there are few or no generally effective mechanisms for directing molecules across the blood brain barrier. Such molecules for transport across the blood brain barrier include, but are not limited to, neurotrophic factors (brain-derived neurotrophic factor, NT-3, NT-4, ciliary neurotrophic factor), growth factors (*e.g.*, nerve growth factor), and the like; antibiotic or antiviral agents, for incipient infections of the brain; and vectors for gene therapy.

Targeting Vectors for Gene Therapy

In yet another embodiment, the present invention provides ligands for targeting DNA vectors to cells that express DEC, in particular, dendritic cells, epithelial cell of the thymus, small intestine, and lung, and brain capillaries. Accordingly, a DNA vector, such as a viral vector, can be modified by conjugation with a DEC ligand for targeting to cells that express DEC. Examples of DNA virus vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kaplitt et al., 1991, Molec. Cell. Neurosci. 2:320-330), an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al. (1992, J. Clin. Invest. 90:626-630), a defective adeno-associted virus vector (Samulski et al., 1987, J. Virol. 61:3096-3101; Samulski et al., 1989, J. Virol. 63:3822-3828), as well as a papillomavirus vector, Epstein Barr virus (EBV) vector, and the like. The viral particles can be modified to include a ligand for DEC, *e.g.*, by chemically cross-linking a DEC ligand to the virus.

Alternatively, the vector can be introduced *in vivo* by lipofection. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding DEC (Felgner, et. al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:7413-7417; *see* Mackey, et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:8027-8031)). The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes (Felgner and Ringold, 1989, Science 337:387-388). The use of lipofection to introduce exogenous genes into the specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. Accordingly, the present invention advantageously provides for targeting a gene for dendritic cells and thymic epithelium by conjugating a DEC-ligand to a liposome vector. Lipids may be chemically coupled to other molecules for the purpose of targeting (*see* Mackey, et. al., 1988, *supra*). Targeted antibodies or glycans could be coupled to liposomes chemically.

It is also possible to introduce the vector *in vivo* as a naked DNA plasmid, preferably by using a DEC ligand as a vector transporter (see, *e.g.*, Wu et al., 1992, J. Biol. Chem. 267:963-967; Wu and Wu, 1988, J. Biol. Chem. 263:14621-14624; Hartmut et al., Canadian Patent Application No. 2,012,311, filed March 15, 1990).

The present invention will be better understood by reference to the following Examples, which are provided by way of exemplification and not limitation.

EXAMPLE 1: DEC-205, A 205 kDa PROTEIN ABUNDANT ON MOUSE DENDRITIC CELLS AND THYMIC EPITHELIUM THAT IS DETECTED BY THE MONOCLONAL ANTIBODY NLDC-145: PURIFICATION, CHARACTERIZATION, AND N-TERMINAL AMINO ACID SEQUENCE

This Example describes the purification and biochemical characterization of the antigen recognized by monoclonal antibody NLDC-145 (Krall et al., 1986, J. Exp.

Med. 163:981). We refer to the protein as DEC-205, in view of its abundant expression by Dendritic and thymic Epithelial Cells, and the observed molecular mass. The protein has been purified at a scale that permits direct biochemical study. The antigen proves to be an integral membrane glycoprotein with a mildly alkaline (pI 7.5) and an electrophoretic molecular mass of 205 kDa, not 145 kDa, as originally reported (Kraal et al., *supra*). About 7 kDa of the mass is contributed by covalently-bound carbohydrates. A panel of plant lectins was used to gain preliminary information on the structures of these glycans. The glycans were then subjected to a variety of exogylcosidase digestions and fluorophore-assisted carbohydrate electrophoresis (FACE) (Jackson, 1990, Biochem. J. 270:705; Jackson and Williams, 1991, Electrophoresis 12:94; Jackson, 1993, Biochem. Soc. Trans. 21:121; Jackson, 1994, Anal. Biochem 216:243). Eight distinct but related biantennary N-linked glycan structures were resolved. These variants differed at their termini, but were based on two fucosylated trimannosyl chitobiose core structures (with and without a bisecting GlcNAc). O-linked glycans were not detected. The amino terminus of the protein is not blocked, and the sequence of its first 25 amino acids is not significantly homologous or similar to any known protein. Two new polyclonal antibodies are described, one raised to the N-terminal peptide sequence, the second to the intact purified protein. On immunoblots, both of these polyclonals recognize a 205 kDa band, which can be specifically depleted by preclearing extracts with NLDC-145.

Materials and Methods

Purification of NLDC-145 and preparation of immunoaffinity resins-- NLDC-145 (rat IgG2a) ascitic fluids were prepared in normal, 6-8 week old, non-SPF, CD2 (BALB/c x DBA/2 F1) female mice (Trudeau Institute) as described (North and Izzo, 1993, J. Exp. Med. 177:1723). The monoclonal was purified by sequential chromatography on immobilized Protein A and Protein G (Pierce). Both the monoclonal and nonspecific rat IgG2a (Zymed) were coupled to resins by reductive amination (AminoLink, Pierce).

Immunoprecipitation -- Bone marrow dendritic cells (BMDC) were prepared from proliferating marrow precursors, as described by Inaba et al. (1992, J. Exp. Med 176:1693). Eight days after the cultures were initiated, 4.8 x 10^7 BMDC were cultured for 1 h in 10 ml of methionine- and cysteine-free medium. Labeling was initiated by adding 1 mCi of (^{35}S)methionine-cysteine (ICN), and cells were collected after 4 h of culture. BMDC were lysed by resuspending them in 700 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 1% ~~Nonidet~~ NONIDET P-40 (Calbiochem), 50 mg/ml BSA (Intergen), with a mixture of protease inhibitors: 5 mM EDTA, 0.5 mg/ml ~~Pefabloc~~ PEFABLOC SC (Boehringer Mannheim), 100 mg/ml PMSF, 5 mg/ml aprotinin, 5 mg/ml pepstatin A and 10 mg/ml leupeptin (the latter 4 inhibitors from Sigma)). Lysates were precleared with 20 mg of rat IgG (Jackson ImmunoResearch), 10 ml of FCS, and 100 ml of packed immobilized Protein G (Pierce). The supernatant was precleared a second time with 100 ml of Protein G, for 1 h. The precleared lysate was divided into 100 ml aliquots. Proteins in two aliquots (6.9 x 10^6 BMDC equivalents) were adsorbed to 50 ml of packed, washed immunoaffinity resin (either NLDC-145 or rat IgG2a), rotating 1 h. Washes were performed as described (Fireston and Winguth, 1990, Methods Enzymol. 182:688). Proteins were analyzed by SDS-PAGE in 10% acrylamide minigels.

Immunoblotting-- SDS-PAGE was performed in 8% acrylamide minigels, 1.5 mm thick. Transfer to nitrocellulose (BA-85, Schleicher and Schuell) was performed at 30 constant volts overnight at 4°C. Filters were blocked in PBS containing 3% (w/v) nonfat dry milk and 0.1% ~~Tween~~ TWEEN 20 for 1 h at room temperature with shaking. Incubation with primary antibodies (0.1-10 μg/ml of purified IgG, ascites or serum diluted 1:1000, or hybridoma supernatant diluted 1:1) was performed in heat-sealed bags for 1 h at room temperature. Filters were washed, then immunostaining was visualized with peroxidase-conjugated F(ab')$_2$ donkey anti-rat or anti-rabbit IgG (Jackson), followed by enhanced chemiluminescence (Amersham).

Purification of DEC-205 from thymi-- The strategy is summarized in Figure 2. Thymi were removed from 50 outbred CD-1 Swiss mice (Taconic) per preparation. Thymi were placed into 50 ml of ice-cold PBS containing 200 mg/ml PMSF and 5 mM EDTA to remove blood, and washed once with the same buffer. Washed organs could be frozen at -20°C. All subsequent purification steps were performed at 0-4°C. Thymi were transferred to a 40 ml Dounce homogenizer (Kimble/Kontes), and resuspended in 30 ml of hypotonic lysis buffer (10 mM Tris-HCl, pH 6.8, with a mixture of protease inhibitors: 5 mM EDTA, 100 mg/ml PMSF, 4 mg/ml aprotinin, 0.5 mg/ml ~~Pefabloc~~ PEFABLOC SC, 4 mg/ml pepstatin A, 10 μg/ml leupeptin). Organs were homogenized with 20 strokes of the loose (0.2 mm clearance) pestle, then 20 strokes of the tight (0.1 mm clearance) pestle. The suspension was left on ice for 20 min, then re-homogenized with an additional 20 strokes of the tight pestle. Nuclei and debris were pelleted by low speed centrifugation (1200 x *g*, 5 min, 4°C). The turbid "postnuclear" supernatant was collected, and the nuclear pellet was washed with 5-8 changes of 15 ml hypotonic lysis buffer, until the supernatant was nearly clear. Supernatants from each wash were pooled. To collect membranes, pooled postnuclear supernatant was centrifuged at 100,000 x *g* for 1 h at 4°C (RC-28S centrifuge, F28/36 rotor, Du Pont-Sorvall). Proteins in the membrane pellet were extracted into 5 ml of hypotonic lysis buffer containing 0.5% (8.3 mM) NP-40. The membrane extract was clarified by a second one-hour, 100,000 x *g* centrifugation.

Clarified membrane extract was precleared by passage over a nonspecific rat IgG column. Nonadsorbed fractions from the preclearing column were pooled, then applied to the NLDC-145 affinity column. Washes were performed in 2 steps: 6 ml (3 bed volumes) of wash-1 (hypotonic lysis buffer with 0.5 M NaCl, without NP-40, substituting 0.5% (17 mM) n-octyl glucoside (Boehringer Mannheim)), then 10 ml of wash-2 (wash-1 without added NaCl). The column was eluted with at least 5 bed volumes of 50 mM glycine-NaOH, pH 11, 0.5% n-octyl glucoside, reducing the maximum flow rate to 10 ml cm^{-2} hr^{-1}. The pH of eluted fractions (1 ml) was adjusted to 7 with 20-30 ml of 2 M glycine-HCl, pH 2. Peak eluates were

pooled and concentrated to <1 ml by ultrafiltration in Centricon-100 units (Amicon) that had been pre-coated with 0.1% SDS, to reduce nonspecific losses to the plastic. Typically, 70-150 μg of DEC-205 could be obtained from 50 thymi.

Isoelectric focusing-- Isoelectric focusing was performed in thin (0.75 mm) slab gels, under denaturing conditions (5.5% acrylamide gels containing 8 M urea, 4% total Ampholine (2:1 ratio of pH 3.5-10 and 5-7, Pharmacia), 0.67% NP-40, 10% glycerol). Samples were focused at 400 constant volts overnight, for a minimum of 6000 volt-hours, at which time the current was less than 1 mA. Lanes were either silver-stained or cut into 0.5 cm sections and eluted into degassed dH_2O for pH gradient measurement.

Detection of glycans-- DEC-205, transferrin (positive control) and creatinase (negative control) were blotted onto nitrocellulose as before. Glycoconjugates were converted to digoxigenin (DIG)-labeled hydrazones after mild nonselective periodate oxidation of *vic*-diols to aldehydes. Staining patterns were visualized with an anti-DIG antibody conjugated to alkaline phosphatase (First CHOice, Boehringer Mannheim).

Chemical deglycosylation-- Two 100 μl samples each of DEC-205 (40 μg) and apotransferrin (100 μg, positive control) were transferred into 0.1% trifluoroacetic acid, 0.05% SDS by G-25 SF spin chromatography, then were lyophilized to dryness. Cleavage was performed with anhydrous trifluoromethanesulfonic acid (Sojar et al., 1987, Methods Enzymol. 138:341) (Oxford GlycoSystems). Polypeptides were separated from cleavage products and excess reagents by TCA precipitation, followed by electrophoresis in 8% acrylamide minigels, adjacent to untreated controls.

Enzymatic deglycosylation-- Peptide-N-glycosidase F from *Flavobacterium meningosepticum* (PNGase F, Boehringer Mannheim) was used to cleave asparagine-linked glycans (Tarentino et al., 1985, Biochem. 24:4665). Aliquots (10

μg per eventual gel lane) of DEC-205 were denatured by boiling for 5 min in the presence of 0.1% SDS. After cooling on ice and brief spinning in a microfuge to collect liquid, 1/5 volume of 5X PNGase buffer (250 mM sodium phosphate, pH 7.0, 50 mM EDTA, 2.5% NP-40, 5% 2-mercaptoethanol) was added, then 1 unit of PNGase F (5 μl). Reactions were incubated overnight at 37°C, then were terminated by adding 1/4 volume of 4X nonreducing SDS-PAGE sample buffer, and boiling for 5 min.

Lectin blotting-- Several digoxigenin-labeled plant lectins (Boehringer Mannheim) were used to stain electroblotted DEC-205 and appropriate positive and negative control glycoproteins. The lectins, their specificities and the concentrations used for staining are summarized in Table 1.

Exoglycosidase digestions and FACE analysis-- N-linked oligosaccharides were released from DEC-205 with PNGase F and labeled with the fluorophore ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) (Jackson, 1990, *supra*; Jackson and Williams, *supra*; Jackson, 1993, *supra*; Jackson, 1994, *supra*). Recombinant exoglycosidases were from Glyko. Electrofluorograms were visualized on an SE1000 FACE workstation (Glyko).

Amino acid sequencing-- DEC-205 was electrophoresed in multiple lanes of 1.5 mm thick 4% minigels prepared using Duracryl (Millipore). Gels were blotted onto polyvinylidene difluoride (PVDF, Bio-Rad). After transfer, filters were soaked for 1 min in 1% acetic acid, stained for 2 min in 0.1% Ponceau S, then were destained briefly in dH_2O. Bands at 205 kDa were excised and submitted for analysis. The N-terminal sequence was aligned to all current databases on the BLAST Internet servers (NCBI, National Library of Medicine, NIH), running the program BLASTP (Altschul et al., 1990, J. Med. Biol. 215:403).

Polyclonal antibodies to intact DEC-205-- Two New Zealand White rabbits (Hazelton) were injected 6 times with the 205 kDa bands cut from Coomassie-

stained, 1.5 mm thick, 4% ~~Duracryl~~ SDS-PAGE gels. Doses ranged from 40-70 μg of stained protein per animal, per injection (4-6 slices), and were given every 3 weeks, with test bleeds (about 15 ml of serum) taken 2 weeks post-injection. For the first injection, slices were emulsified in Complete Freund's adjuvant (CFA) and injected intradermally into multiple sites on the back. Incomplete Freund's (IFA) was the adjuvant for boosts. Responses were monitored by Western blotting crude thymic membrane extracts with graded doses of serum. Animals were boosted further with the unfractionated eluate from the immunoaffinity column, i.e., soluble protein rather than gel slices. Four boosts, averaging 50 μg per injection, were given to both rabbits. IgG fractions were prepared by Protein A chromatography.

Polyclonal antibodies to the N-terminal peptide-- The hapten-coupling strategy focused on the lone cysteine at residue 19 (Figure 6A). Peptide N1 (SESSGNDPFTIVHENTGKC) (SEQ ID NO: 2) was coupled to keyhole limpet hemocyanin (KLH) and ovalbumin (OVA) using maleimide chemistry (Imject, Pierce). An average of about 250 peptides were conjugated to each molecule of KLH, and about 6 peptides per molecule of OVA. The KLH-peptide conjugate was divided into aliquots of 400-500 μg each, and was injected eight times into two New Zealand White rabbits (200-250 μg per injection), again emulsifying into CFA for the initial immunization and IFA for boosts. To remove any anti-KLH reactivity from the sera, they were precleared on a KLH-cysteine column. Anti-peptide antibodies were isolated on a peptide-OVA column, where the peptide was coupled to an irrelevant carrier.

Results

NLDC-145 immunoprecipitates and Western blots an antigen of 205 kDa, not 145 kDa-- To determine the molecular mass of the NLDC-145 antigen, DCs generated from proliferating bone marrow progenitors *in vitro* (Inaba et al., *supra*). This method provided abundant NLDC-145 (+) DCs in high purity. On day 8 of culture, the DCs were metabolically labeled with [^{35}S]methionine-cysteine. Extracts were immunoprecipitated using either immobilized NLDC-145 or control

nonspecific rat IgG2a. Autoradiography of the precipitates after reducing SDS-PAGE (Figure 1A) revealed that NLDC-145 bound a single specific band with an apparent mass slightly greater than 200 kDa (myosin marker), not 145 kDa, as originally reported (Kraal et al., *supra*). Numerous nonspecific bands were also visible in both lanes, including a prominent band at 45 kDa, presumably G-actin (Fosman, 1976, *Handbook of Biochemistry and Molecular Biology, Volume 1:Proteins*, CRC Press:Cleveland, Ohio).

To verify this measurement, NLDC-145 was used as the probe in a Western blot. Five thymi from 8 week-old BALB/c mice were homogenized in 2 ml of the same lysis buffer used for immunoprecipitation. Graded doses of clarified thymic extract were electrophoresed under nonreducing and reducing conditions, and blotted to nitrocellulose. NLDC-145 bound a single major band (Figure 1B) that co-migrated with the *prestained* myosin marker at 205 kDa, confirming the estimate made by immunoprecipitation (above). Under nonreducing conditions (Figure 1B, left filter), as few as 7 x 10^{-4} thymic equivalents could be clearly visualized with this dose of monoclonal NLDC-145 IgG. However, after reduction of disulfides in the crude extract with mercaptoethanol (right strip), the mAb failed to stain, even at the highest dose of lysate. We concluded that the antigen recognized by NLDC-145 has a mass of 205 kDa, and that the epitope detected by the mAb requires an intramolecular disulfide bond.

Purification of DEC-205-- The epithelial cells of the thymic cortex express the antigen abundantly (Kraal et al., *supra*), even more abundantly than the less-numerous DCs in the thymic medulla. Therefore, a scheme to isolate the protein from thymi was developed (Figure 2). Briefly, thymi were homogenized under hypotonic lysis conditions, in the presence of a "cocktail" of high doses of 6 protease inhibitors. Membranes were isolated and extracted with NP-40. After clarification, the extract was precleared on a rat IgG column, then chromatographed on the NLDC-145 column.

SDS-PAGE analysis of purified, concentrated DEC-205 (Figure 3A) revealed a predominant 205 kDa band and only trace contaminant bands on Coomassie staining. Silver staining exposed a large number of lower molecular mass contaminants, dictating the need for further purification by preparative electrophoresis before amino acid sequencing.

In an effort to follow step yields during the purification process, key fractions were diluted to the same volume (the volume of the post-nuclear supernatant), and immunoblotted (Figure 3B). Because the fractions were isovolumic, staining intensities on the filter should reflect the relative concentrations of DEC-205 at each phase of the separation. Roughly 30% of the protein appeared to be lost to the nuclear pellet (lane 1) in the first step, hypotonic lysis. Another 10% or so (lane 3) failed to sediment with the membrane pellet (lane 4) during the first high-speed centrifugation. A constant small fraction -- perhaps 5-10% cumulatively -- passed through the NLDC-145 column, in both early and late nonadsorbed fractions (lanes 6 and 7). The cumulative yield in the final eluate (lane 8) was therefore about 50%.

When the eluate was intentionally overloaded at five times the isovolumic concentration (lane 10), a "ladder" of at least 6 smaller, minor bands was clearly and consistently seen. All of these bands must contain the NLDC-145 epitope. They ranged down in rather orderly fashion (i.e., not a continuous "smear") to a fairly intense 80 kDa component. Careful inspection of earlier fractions (in particular, lanes 2, 3, 4, and 8) revealed that these minor bands were present from the earliest stages in the isolation. Presumably, these bands were produced by proteolytic degradation of the intact 205 kDa protein.

DEC-205 is an integral membrane protein with an isoelectric point of 7.5-- To determine whether the 205 kDa protein was an integral membrane protein, thymic membrane pellets (prepared as in Figure 2) were resuspended in: (1) hypotonic lysis buffer containing 0.5% NP-40 (as usual); (2) the same buffer containing 1 M

KCl instead of detergent; or (3) 100 mM Na_2CO_3 pH 11.5, containing all 6 protease inhibitors used in routine purifications. After one hour of gentle mixing, suspensions were clarified (100,000 x *g*, 60 min, 4°C), and supernatants were collected. Precipitates from the high-salt and high-pH extractions were then resuspended in hypotonic lysis buffer with 0.5% NP-40, and the extraction and clarification steps were repeated. A Western blot of the five extracts generated this way (Figure 4A) revealed that DEC-205 could not be released from the membrane pellet under conditions of either high ionic strength (lane 2) or extreme pH (lane 3). Detergent was required for its solubilization (lanes 1, 4 and 5). DEC-205 is therefore an integral membrane protein (Fujiki et al., 1982, J. Cell Biol. 93:97).

Isoelectric focusing was performed in slab gels under denaturing conditions with silver staining. A relatively homogeneous isoelectric point was observed at pH 7.5 (Figure 4B). A sharply focused central band at that pH was consistently bordered by a narrow "fringe" of fainter staining, extending from pH 7.4 to pH 7.6.

DEC-205 is a glycoprotein, bearing heterogeneous N-linked glycans -- To determine whether DEC-205 was glycosylated, purified 205 kDa protein was electrophoresed on a gel which also contained samples of transferrin (a known glycoprotein) and creatinase (a known nonglycoprotein), and was electroblotted to nitrocellulose. Filters were oxidized with sodium *meta*-periodate at room temperature, nonselectively converting vicinal diols in carbohydrates to aldehydes. A digoxigenin (DIG)-tagged hydrazide was applied to the filter, converting the aldehydes to DIG-hydrazones. The filter was blocked, and then covalently-bound DIG was detected by staining with an anti-digoxigenin antibody conjugated to alkaline phosphatase. The staining pattern (Figure 5A) revealed that DEC-205 (lane 2) is a glycoprotein, like transferrin (lane 1).

To determine how much of the apparent molecular mass was contributed by glycans, the purified protein was chemically deglycosylated. Anhydrous trifluoromethanesulfonic acid (TFMSA) does not attack the primary structure of

proteins, yet hydrolyzes both asparagine-linked and serine/threonine-linked glycans at their points of attachment to amino acid sidechains (Sojar et al., *supra*; Dabich et al., 1993, Biochem. Biophys. Acta 1164:47). Upon TFMSA treatment (Figure 5B), both apotransferrin (lane 2) and DEC-205 (lane 4) exhibited increased electrophoretic mobility compared to untreated samples (lanes 1 and 3). Linear regression analysis of the migratory distances of the treated samples revealed that, as expected, deglycosylated apotransferrin lost 5 kDa in apparent molecular mass (MacGillivray et al., 1983, J. Biol. Chem. 258:3543), while DEC-205 lost roughly 7 kDa. This 7 kDa shift was consistent with the removal of two to three complex-type N-linked glycans from the 205 kDa protein. An uncertain number of smaller O-linked glycans might also have been removed.

To begin to define the types of carbohydrate residues present on the protein, blotted purified material was probed with a panel of digoxigenin-labeled plant lectins. Asparagine-linked glycans were removed from an aliquot of DEC-205 by treating it with peptide N-glycosidase F (PNGase F). Treated and untreated protein was blotted to nitrocellulose along with positive and negative control glycoproteins. After confirming transfer by staining the filters with Ponceau S (not shown), membranes were blocked and stained with DIG-lectins, used at the concentrations listed in Table 1.

TABLE 1

Table 1: Staining of electroblots with digoxigenin-labeled plant lectins

Lectin	Source	Specificity	Concentration (μg/ml)	Staining on PNGaseF-treated DEC-205	Staining on undigested DEC-205
SNA	*Sambucus nigra*	NANAα2-6Gal/GalNAc	1	(-)	(-)
MAA	*Maackia amurensis*	NANAα2-3Gal	5	(-)	(-)
PNA	*Arachis hypogaea*	Galβ1-3GalNAc	10	(-)	(-)
DSA	*Datura stramonium*	Galβ1-4GlcNAc, GlcNAc-Ser/Thr	1	(-)	strong (+)
AAA	*Aleuria aurantia*	L-Fucα1-6GlcNAc	1	(-)	strong (+)
GNA	*Galanthus nivalis*	Manα1-3Man (α1-3 > α1-6 > α1-2)	1	(-)	very weak (+) at 200 kDa

Lectin staining patterns (Table 1) showed that the N-linked glycans lacked N-acetyl neuraminic acid in either of its two most common linkages to galactose, α2-6 and α2-3, since the protein failed to bind lectins SNA and MAA, respectively. The core disaccharide of O-linked glycans was not present in unsubstituted form, since PNA did not bind. Pretreatment of the protein with neuraminidase did not render it stainable with PNA (not shown), so any O-glycans present were not capped with sialic acid. If present, they are few in number, since selective removal of N-linked glycans with PNGase F reduced the protein's apparent mass by 7 kDa (not shown), just as nonselective chemical deglycosylation did. Undigested DEC-205 stained intensely with DSA, and staining was ablated by PNGase F digestion. Thus, one or more of the N-glycans terminates with Galβ1-4GlcNAc. Terminal fucose linked α1-6 to GlcNAc is also present on at least one of the N-glycans, since the undigested protein stained strongly with AAA. Lectin GNA weakly stained a band with a mobility slightly greater than the DSA (+) and AAA (+) bands. Presumably this 200 Kda band, containing high-mannose N-linked glycans, represented a subpopulation of newly-synthesized molecules which had not yet undergone oligosaccharide processing reactions in the Golgi complex.

The structures of the N-linked glycans on DEC-205 were further defined by fluorophore-assisted carbohydrate electrophoresis (FACE). PNGase F released 8 different N-linked glycan structures from DEC-205, with electrophoretic migrations ranging from 5.1 to 10.1 glucose units (Figure 5C). The glycan yield was too low to permit excision and sequencing of each of the 8 individual bands, so the mixture was subjected to analysis with exoglycosidases (Figure 5D). Digestion with α-galactosidase (lane 2) simplified the pattern, indicating that some of the glycans terminate with Galα1-(1 or 2)Gal. Addition of NANase III (lane 3, specific for α2-3, α2-6 and α2-8 linked N-acetylneuraminic acid) simplified the pattern still further (loss of sialic acid reduces band mobility), demonstrating that some of the glycans terminate with sialic acid. Neither NANase I (selective for α2-3 linkages) nor NANase II (selective for both α2-3 and α2-6 linkages) altered the pattern of bands (not shown). This was consistent with the lack of staining by lectins SNA and MAA (above), and demonstrated that the terminal sialic acid present on certain

DEC-205 glycans is linked α2-8. Further treatment with β-galactosidase (lane 4) produced a loss of two galactoses (consistent with DSA staining and a biantennary structure), and reduced the complexity of the pattern to a doublet. On addition of β-N-acetylhexosaminidase to the enzyme mixture (lane 5), the lower band of the doublet released 2 GlcNAc to yield a band characteristic of a fucosylated trimannosyl chitobiose core. However, the upper band resisted digestion, suggesting the presence of either a "bisecting" GlcNAc linked to a trimannosyl core between the antennas, or possibly a branching fucose. The bisecting GlcNac was revealed by the fact that the upper band could be at least partially digested (2-fold decrease in fluorescence intensity) when the enzyme concentration was doubled (lane 6). Further digestion with α-mannosidase (lane 7) was incomplete, mostly releasing a single mannose (major band), but showed the beginning of release of a band that co-migrated with the fucosylated mannosylchitobiose core structure (lane 9, "FC"). Doubling the amount of α-mannosidase and adding an α-fucosidase specific for fucose linked α1-2,-3,-4 and -6 to GlcNAc (lane 8) led to essentially complete digestion, releasing a band that co-migrated with the non-fucosylated mannosylchitobiose core (lane 9, "C").

In combination, lectin staining and FACE analysis demonstrated that DEC-205 contains biantennary N-linked glycans with two kinds of fucosylated core structures, one with a bisecting GlcNAc, one without (Figure 5E). Further heterogeneity is introduced at the outer ends of these structures, which terminate with either α-linked galactose, β1-4 linked galactose or α2-8-linked sialic acid, in a total of 8 different permutations.

N-terminal amino acid sequence, and polyclonal antibodies to the N-terminal peptide and intact DEC-205-- Purified DEC-205 was submitted for amino-terminal sequencing, and the first 25 residues were identified unambiguously (Figure 6A). When the sequence was aligned against all available protein sequence databases, no significant homologies were found. In order to verify that the 205 kDa protein we had purified was the antigen recognized by NLDC-145, a 19-residue synthetic N-terminal peptide (Figure 6A, first 19 amino acids) was synthesized, purified,

coupled to keyhole limpet hemocyanin, and injected into a pair of rabbits. Peptide-reactive antibodies from hyperimmune sera were purified on an affinity resin prepared by coupling the peptide to the irrelevant carrier ovalbumin. In parallel, purified DEC-205 was injected into a second pair of rabbits, and hyperimmune IgG was purified by Protein A chromatography. On immunoblots, both polyclonals stained a 205 kDa band in crude extracts, like NLDC-145 (Figure 6B, 'extract' lanes). The affinities of both polyclonals for the blotted 205 kDa protein were roughly 100 times higher than the monoclonal. Here, 0.1 μg/ml of either polyclonal gave a staining intensity comparable to that obtained with 10 μg/ml of NLDC-145 IgG. Both polyclonals bound "ladders" of minor bands, similar to those seen with the mAb. Staining of the 205 kDa band was specifically ablated when the extracts were precleared with immobilized NLDC-145 (Figure 6B, 'depleted' lanes), but was restored on proteins eluted from the preclearing resin (Figure 6B, 'eluate' lanes), confirming that the correct protein had been purified and sequenced.

Discussion

A purification method based on immunoaffinity chromatography was used to isolate the antigen bound by the mAb NLDC-145, an antigen which was reported to be expressed at high levels by murine dendritic cells and thymic epithelium (Kraal et al., *supra*). As a protein source, whole murine thymi were lysed rather than attempting the daunting task of purifying large numbers of DCs. The isolated protein was about 95% pure, and was obtained with a yield in the hundred-microgram range, sufficient for N-terminal amino acid sequencing and basic biochemical studies. It would have required approximately 10^9-10^{10} DCs to provide a comparable amount of protein.

The electrophoretic molecular mass of the protein was consistently 205 kDa. In the original report by Kraal et al (*supra*), NLDC-145 was used to immunoprecipitate detergent extracts from surface-iodinated low-density lymph node cells. Only a single serine protease inhibitor, 1 mM PMSF, was present in the lysis buffer. A

single predominant labeled protein was bound, with an apparent molecular mass of 145 kDa under both reducing and nonreducing electrophoresis conditions, leading the authors to append the number "145" to the clone's name. When Puré et al. (1990, J. Exp. Med. 172:1459) attempted to reproduce the immunoprecipitation, they prepared detergent extracts of (^{35}S)methionine-labeled cultured epidermal DCs (Langerhans cells), using a lysis buffer that contained multiple serine protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin and 0.1% DIFP). They resolved a protein with an apparent molecular mass of > 200 kDa under reducing conditions.

In this Example, immunoprecipitation of the antigen from bone marrow dendritic cells in the presence of high doses of inhibitors directed not only to serine proteases, but also to sulhydryl, aspartic and metalloproteases, yielded a protein of an apparent mass of > 200 kDa, in agreement with Puré et al. The mAb could be used to stain Western blots under nonreducing conditions, and measured a mass of 205 kDa independently by this method. The protein that eluted from the NLDC-145 immunoaffinity column had a mass of 205 kDa, and the amino terminus of this protein revealed a sequence with no significant homology or similarity to any other protein currently in the databases. In order to prove that the 205 kDa purified protein was the antigen detected by NLDC-145, polyclonal antibodies to the N-terminal peptide and to the intact purified protein were prepared. Both polyclonals stained a 205 kDa band on immunoblots. This staining could be eliminated by pretreating extracts with NLDC-145, demonstrating that the correct protein had been purified and sequenced. Thus, the 205 kDa protein purified here is the authentic antigen recognized by the NLDC-145 monoclonal antibody. We believe that the lysis conditions employed by Kraal et al. (*supra*), with minimal antiproteolytic coverage, may have permitted limited degradation of the protein.

We propose the name "DEC-205" for the protein, to indicate its high-level expression by Dendritic and thymic Epithelial Cells, and to revise the prior estimate of its electrophoretic molecular mass. DEC-205 is an integral membrane glycoprotein, bearing 2-3 biantennary complex-type N-linked glycans that comprise

about 7 kDa of the overall electrophoretic molecular mass. These glycans are built on two different core structures, and vary further at their termini, to produce 8 variants, some of which contain sialic acid. Nevertheless, on electrofocusing, the isoelectric point of DEC-205 is slightly alkaline (pH 7.5), suggesting that the protein may be relatively rich in basic residues. The pI is fairly homogeneous: a faint-staining, narrow "fringe" of protein surrounds the main pI, but extends only from pH 7.4 to 7.6, reflecting limited heterogeneity of charge. Considering the large overall mass of the protein and the relative paucity of bound carbohydrates, the sialylated glycan variants detected should not perturb the pI of DEC-205 excessively.

DEC-205 is very sensitive to proteolytic degradation. Precautions had to be taken to inhibit a broad range of proteases during the purification, and to remove the cytosolic fraction after hypotonic lysis, or else the yield was very low. Proteolysis appears to proceed by a distinctly nonrandom pathway. Despite the continuous presence of high concentrations of six protease inhibitors in our buffers, we invariably observed a "ladder" of 6-8 discrete, minor, lower molecular mass bands, ranging down to about 80 kDa and containing the NLDC-145 epitope, whenever the antigen was blotted at high levels. This orderly array of proteolytic fragments could be observed in every preparation blotted, from crude thymic membrane extract (best seen in Figure 4, but also present in Figures 1B and 3B) to the purified, ultrafiltered immunoaffinity eluate (Figure 3B). The "fringe" of staining around the main isoelectric point is likely to be at least partly produced by these relatively large proteolytic fragments.

The large size, nonrandom distribution and relative protease resistance of the "ladder" of minor bands suggests to us that the complete primary structure of DEC-205 will reveal a modular architecture, with multiple protease-resistant domains joined by more protease-sensitive connecting segments.

EXAMPLE 2: DEC-205, A RECEPTOR EXPRESSED BY DENDRITIC CELLS AND THYMIC EPITHELIAL CELLS, HAS TEN C-TYPE LECTIN DOMAINS AND IS INVOLVED IN ANTIGEN PROCESSING

This example reports that DEC-205 is a receptor with ten C-type lectin domains which is homologous to the macrophage mannose receptor (MMR), and other related receptors that bind carbohydrates and mediate endocytosis. The function of DEC-205 was investigated with monoclonal and polyclonal anti-DEC-205 antibodies. It was determined that DEC-205 on dendritic cells is rapidly internalized via coated vesicles, and delivered to a multivesicular endosomal compartment that resembles the MHC class-II containing vesicles implicated in antigen processing. Furthermore, rabbit anti-DEC-205 antibodies were efficiently processed by dendritic cells and presented to rabbit IgG specific T cell clones. These experiments suggest that DEC-205 is a novel endocytic receptor that can be used by dendritic cells and thymic epithelial cells to direct captured antigens from the extracellular space to a processing compartment.

Materials and Methods

Purification of dendritic cells-- Dendritic cells from 7 day bone marrow cultures were treated with polyclonal rabbit anti-DEC-205 F(ab)'2 fragments and 10 nm gold-labeled goat anti-rabbit IgG as described in Figure 3 and processed for electron microscopy. For each time point 10 grids were examined, and all cells that were labeled with gold were photographed. gold particles were counted and scored by a blinded observer based, on standard morphological criteria. the numbers in parentheses represent the percentage of total gold particles scored in each compartment.

Northern blotting-- For Northern blots, 2 μg of mRNA were electrophoresed in 0.8% agarose formaldehyde gels. Samples were transferred to nylon membranes and probed with an anti-sense RNA probe that spanned nucleotide positions 3688-5200 in the DEC-205 cDNA. The blot was subsequently stripped and re-

hybridized with glyceralaldehyde-3-phosphate dehydrogenase probe as a loading control.

Electron microscopy-- Dendritic cells harvested from 7 day mouse bone marrow cultures were incubated with 10 μg/ml of either: polyclonal anti-DEC-205, Fab'2 fragments of polyclonal anti-DEC-205; or biotinylated monoclonal NLDC-145, on ice for 30'. Excess primary antibody was removed by washing cells 3 times with RPMI-1640, 10% FCS, 0.02% NaN3. The cells were then incubated for 30' on ice with either: a 1:5 dilution of 10 nm gold labeled goat anti-rabbit IgG; or a 1:5 dilution of 10 nm gold labeled streptavidin respectively. Excess secondary reagent was removed by washing cells as above. Dendritic cells were then either fixed with 2.5% glutaraldehyde for a time-zero point, or incubated for the stated times at 37°C before fixation and processing for electron microscopy.

Antigen presentaion-- 105 BALB/c mouse Dendritic cells obtained from day 7 bone marrow cultures were co-cultured with 105 2R.50 rabbit IgG specific T hybridoma cells for 48 hours in triplicate (Boom et al., J Exp Med 1988 167:1350-64). The supernatants were assayed for IL-2 concentration using the HT-2 indicator cell line. IL-2 production by the 2R.50 cells is plotted against the concentration of antibody in the cultures on a log scale. The error bars indicate the standard deviation from the mean.

Results and Discussion

This Example reports the molecular characterization of the 205 kDa cell surface protein described in Example 1. Using oligonucleotide probes based on the protein sequence, fourteen cDNA clones were obtained from three separate thymic and dendritic cell cDNA libraries. All of the cDNA clones were derived from the same mRNA. Clones containing the putative 5′ end of the DEC-205 cDNA encoded the N-terminal peptide of the DEC-205 antigen, and this was preceded by a hydrophobic leader consistent with a signal sequence. The composite cDNA had a single 5.2 Kb open reading frame encoding a protein of 1,722 a.a. with a predicted

molecular weight of 195 Kda that included all 29 unambiguous DEC-205 peptide sequences (Figure 1). A 7.5 Kb mRNA that corresponds to this cDNA was expressed at high levels in dendritic cells, thymus, and lymph nodes, a pattern that corresponds to that which was obtained by staining tissues with the NLDC-145 monoclonal antibody (Kraal et al., *supra*) (Figure 8).

The sequence of DEC-205 was aligned with known proteins in the database, and it was determined that it is homologous to the macrophage mannose receptor (MMR) (Taylor et al., 1990, J. Biol. Chem. 265:12156-62; Ezekowitz et al., 1990, J. Exp. Med. 172:1785-94) and the phospholipase A2 (PLA2) receptors of rabbit skeletal muscle (Lambeau et al., 1994, J. Biol. Chem. 269:1575-8), and to bovine pancreas (Ishizaki et al., 1994, J Biol Chem. 269:5897-904) (Figure 1). All known members of this family, which has been designated as the group VI C-type animal lectins (Drickamer and Taylor, 1993, Annu. Rev. Cell Biol. 9:237-64), are type 1 trans-membrane proteins with short cyctoplasmic domains that mediate receptor endocytosis (Ezekowitz et al., 1990, J. Exp. Med. 172:1785-94; Ishizaki et al., 1994, J. Biol. Chem. 269:5897-904; Taylor et al., 1992, J. Biol. Chem. 267:1719-26). The extracellular portions of MMR family proteins have a distinctive cysteine-rich N terminal domain, followed by a fibronectin type II repeat, and eight Ca^{++} dependent carbohydrate recognition domains (C-type CRDs) (Taylor et al., 1990, J. Biol. Chem. 265:12156-62; Lambeau et al., 1994 J. Biol. Chem. 269:1575-8; Ishizaki et al., 1994, J. Biol. Chem. 269:5897-904). DEC-205 diverges from this pattern only in that it has ten instead of the usual eight C-type CRDs (Figure 1A and 1B). The functions of the cysteine-rich domain and the fibronectin repeat in group VI lectins have not been defined. By contrast, there is extensive experimental evidence that the C-type CRDs are carbohydrate-binding domains (reviewed by (Drickamer and Taylor, 1993, Annu. Rev. Cell Biol. 9:237-64)) and both the MMR and rabbit PLA2 receptor bind carbohydrates (Lambeau et al., 1994, J. Biol. Chem. 269:1575-8; Drickamer and Taylor, 1993, Annu. Rev. Cell Biol. 9:237-64). The carbohydrate contact residues defined for the C-type CRDs in rat serum mannose-protein and E-selection (Drickamer, 1992, Nature 360:183-86; Weis

et al., 1991, Science 254:1608-15; Weis et al., 1992, Nature 360:127-34; Graves et al., 1994, Nature 367:532-8) are not conserved in DEC-205. However, additional mechanisms must exist for carbohydrate binding by C-type CRDs since sequence features initially defined as "essential" for carbohydrate binding are also absent from the CRDs of NKR1, and rabbit PLA2 receptors which bind avidly to carbohydrate ligands (Lambeau et al., 1994, J. Biol. Chem. 269:1575-8; Bezouska et al., 1994, Nature 372:150-57). CRDs are found in over one hundred other proteins (Drickamer and Taylor, 1993, Annu. Rev. Cell Biol. 9:237-64), but the CRDs in DEC-205 are most closely related to those found in the bovine and rabbit PLA2 receptors, and the MMR (34.6% identity with bPLA2 receptor and 26.7% identity with hMMR). Indeed there is an ordered correspondence between CRDs 1-5 and 7-8 in DEC-205 and CRDs 1-5 and 7-8 in other group VI animal lectins, whereas CRD 6 of DEC-205 most closely resembles the first CRDs in other family members. The unusual CRDs in DEC-205, numbers 9 and 10, are most closely related to CRDs 7 and 8 in other group VI lectins, and may have arisen during a gene duplication event. At least two of the CRDs in the MMR are known to bind mannose (Taylor et al., 1992, J. Biol. Chem. 267:1719-26), and grouping of several CRDs increases the affinity of the MMR for carbohydrate ligands (Taylor and Drickamer, 1993, J. Biol. Chem. 268:399-404. The same mechanism may be utilized by DEC-205 to increase both the affinity and diversity of carbohydrates bound by this receptor.

The receptor's function was investigated using a combination of monoclonal and polyclonal anti-DEC-205 antibodies. The observation that the cytosolic domain of DEC-205 contains conserved aromatic amino acids (Figure 7), which have been implicated as part of an endocytic motif (Ezekowitz et al., 1990, J. Exp. Med. 172:1785-94; Ishizaki et al., 1994, J. Biol. Chem. 269:5897-904; Chen et al., 1990, J. Biol. Chem. 265:3116-3123; Collawn et al., 1990, Cell 63:1061-72), suggested that this receptor might be used by dendritic cells and thymic epithelial cells to deliver a variety of extracellular glycoprotein antigens to an intracellular antigen processing compartment. To test this idea, the fate of immunogold-labeled

monoclonal and polyclonal anti-DEC-205 antibodies bound to DEC-205 on dendritic cells was examined by electron microscopy in a time course experiment (Figure 9). Similar results were obtained using the monoclonal antibody and intact or F(ab')2 fragments of polyclonal antibodies in two separate experiments. At time zero, 95% of the cell-associated gold particles were found on the plasma membrane, After warming the sample to 37°C for only one minute, 38% of the particles were found in coated vesicles or coated pits. By 20 minutes after crosslinking, 79% of the gold particles were in a multivesicular compartment that is characteristic of dendritic cells (Steinman et al., 1979 J. Exp. Med. 149:1-16; Kleijmeer et al., 1994, J. Invest. Dermatol. 103:516-523) and resembles the MHC class-II containing vesicles that are thought to be involved in antigen processing (Amigorena et al., 1994, Nature 369:113-120; Qiu et al., 1994, J. Cell Biol. 125:595-609; West et al., 1994, Nature 369: 147-151; Tulp et al., 1994, Nature 369:120-26; Schmid and Jackson, 1994, Nature 369:103-4) (Figure 9 and Table 2). Thus, DEC-205 is rapidly internalized via coated vesicles, and antibodies bound to the internalized receptor are delivered to multivesicular endosomal compartment.

TABLE 2

Compartmental localization of gold particles by electron microscopy

	Plasma Membrane	Coated Pit/Vesicle	Multi-vesicular Endosome	Lysosome
0'	156 (95%)	8 (5%)	0	0
1'	68 (62%)	31 (38%)	0	0
5'	129 (41%)	83 (28%)	99 (32%)	0
20'	12 (5%)	3 (1%)	175 (79%)	32 (14%)
60'	17 (7%)	9 (5%)	46 (19%)	172 (70%)

To determine whether DEC-205 could deliver antigen to an active antigen processing compartment, dendritic cells were treated with rabbit anti-DEC-205

antibodies, and assayed for presentation of rabbit IgG-peptide/MHC complexes to T cell clones (Boom et al., 1988, J. Exp. Med. 167:1350-64). Negative controls included non-specific rabbit antibodies, and rabbit antibodies to IgG2a that are efficiently presented by B cell lines (Boom et al., 1988, J. Exp. Med. 167:1350-64). It was determined that dendritic cells presented rabbit anti-DEC-205 to the T cells clones two orders of magnitude more efficiently than the non-specific rabbit antibodies or rabbit anti-IgG2a (Figure 10). Thus, DEC-205 resembles membrane immunoglobulin on B cells in that the crosslinked receptor is efficiently internalized and bound ligands are delivered to an intracellular compartments that are active in antigen processing (Chestnut and Gray, 1981, J. Immunol. 126:1075-79; Rock et al., 1984, J. Exp. Med. 160:1102-25; Lanzavecchia, 1985, Nature 314:538-39).

In conclusion, dendritic cells and thymic epithelial cells express a novel receptor, DEC-205, which contributes to antigen presentation. The multi-lectin domain structure suggests that this receptor can be used by dendritic cells and thymic epithelial cells to capture and endocytose diverse carbohydrate bearing antigens and direct them to an antigen processing compartment.

EXAMPLE 3: EXPRESSION OF THE DEC-205 ON DENDRITIC CELLS AND OTHER SUBSETS OF MOUSE LEUKOCYTES

Prior studies by a variety of groups demonstrated that the mAb NLDC-145 reacted primarily with dendritic cells (DCs) and the epithelial cells of the thymic cortex. As shown in Example 1, this mAb recognizes DEC-205, a 205 kDa integral membrane glycoprotein with a unique amino-terminal sequence, and a rabbit polyclonal antibody to purified DEC-205 with higher affinity for the blotted antigen than the original mAb was generated. Both the polyclonal and NLDC-145 antibodies have been used in this Example to reassess the expression and function of DEC-205 on leukocytes. Cytofluorography revealed that DCs derived from the epidermis (Langerhans cells) and from proliferating bone marrow progenitors (BMDCs) expressed high levels (2-3 logs) of DEC-205, while freshly-isolated

spleen DCs comprised two subsets, most (80%) staining at low levels ($\leq$ 1 log), the remainder moderately (1.5 logs). DEC-205 epitopes were sensitive to trypsin, but were regenerated in culture. Resident and inflammatory peritoneal macrophages did not express the antigen, except for small amounts on thioglycollate-elicited cells. B cells from spleen, lymph node, bone marrow, blood and peritoneal fluid expressed levels of DEC-205 that were 10 to 50-fold lower than those on BMDCs. Marrow pro- and pre-B cells did not express DEC-205. Polyclonal anti-DEC-205 failed to inhibit either stimulation of a primary mixed leukocyte reaction by DCs *in vitro*, or a local graft vs. host response *in vivo*, where parental T cells were injected into F1 mice. DEC-205 is therefore more broadly expressed on leukocytes than previously appreciated.

The monoclonal antibody described in Kraal et al. (1986, J. Exp. Med. 163:981) was not able to block any DC functions tested, either *in vitro* or in mice given long-term injections of NLDC-145 IgG from birth (Breel et al., 1988, Immunol. 63:331). Because of the unique tissue distribution of the antigen recognized by NLDC-145, and because of its abundant expression on a cell type (dendritic cells) for which few restricted mAbs have been identified, the cell specificity and potential function of DEC-205 was reexamined. The new polyclonal antibody (described in Example 1) was used to improve detection of DEC-205, and to attempt to perturb DC function.

Materials and Methods

Mice-- Adult (6 to 10 wk old) mice of both sexes were studied, including (C57BL/6 x DBA/2) and (BALB/C x DBA/2) F1 mice from the Trudeau Institute (Saranac Lake, NY), and (C57BL/6 x BALB/C) F1 and BALB/C mice from Japan SLC (Hamamatsu, Shizuoka, Japan).

Cell Suspensions-- Cells were studied either immediately after isolation from the animal or following a period of culture in RPMI-1640 medium supplemented with 5% FCS, 50 μM 2- mercaptoethanol, and 20 μg/ml gentamicin. Spleens, thymi,

and lymph nodes were either teased with forceps, or additionally digested with collagenase (Swiggard et al., 1992, *In Current Protocals in Immunology*, Coligan et al., (Eds), Green Publishing Associates and Wile Interscience: New York Supplement 3, pp. 3.7 1-11; Crowley et al., 1989, Cell Immunol. 118:108), with similar results. Bone marrow cells were flushed with a syringe from femurs and tibias, while blood was obtained by cardiac puncture in heparin. All cell suspensions were depleted of red cells by lysis in 0.83% ammonium chloride solution. Dendritic cells were obtained from three sources, each as described: the epidermal sheets of mouse ears (Schuler and Steinman, 1985, J. Exp. Med. 161:526), the low density plastic adherent population of spleen (Saveggard et al., *supra*; Crowley et al., *supra*), and proliferating bone marrow progenitors that were expanded in rGM-CSF (Inaba et al., 1992, J. Exp. Med. 176:1693). Peritoneal cells were either resident populations or were elicited by various inflammatory stimuli: proteose peptone 3 days earlier, thioglycollate broth 4 days earlier, 50 μg concanavalin A 2 days earlier, or 10^7 live *Mycobacterium bovis* BCG organisms 7 days earlier. Several populations were also studied after a period of 1-3 days in culture. The B cells in lymph node suspensions were stimulated with the B cell mitogens lipopolysaccharide (10 μg/ml LPS, from *E. Coli* 0111:B4, Difco, Detroit, MI), anti-IgM plus IL-4 (10 μg/ml goat $F(ab')_2$ anti-mouse IgM, Jackson ImmunoResearch (West Grove, PA), plus 50 U/ml of recombinant murine IL-4, kind gift of Dr. T. Sudo, Basic Research Laboratories, Toray Industries, Kamakura, Japan), and CD40 ligand (CD40L: L cells transfected with CD40L (kind gift of Dr. H. Yagita, Juntendo University School of Medicine, Japan), fixed with 1% paraformaldehyde and washed 3 times in PBS before 1:1 coculture with B cells). Dendritic cells in skin were cultured as whole epidermal suspensions and then enriched by flotation on dense bovine albumin (Schuler and Steinman, *supra*), while dendritic cells in spleen were cultured from low density spleen adherent cells, with or without supplementation in rGM-CSF (200 U/ml) or keratinocyte-conditioned medium, as described (Witner-Pack et al., 1987, J. Exp. Med. 166:1484).

Two-color labeling methods were used to simultaneously identify a particular subset of leukocytes (PE-labeled antibody) and DEC-205 (NLDC-145 rat mAb or rabbit polyclonal anti-DEC-205 followed by FITC labeled anti-Ig). Nonreactive control antibodies were nonimmune rat IgG2a (Zymed, South San Francisco, CA) and rabbit IgG (Jackson ImmunoResearch; intact IgG or $F(ab')_2$ prepared by us). The staining sequence was: (a) primary anti-DEC-205 or nonimmune; (b) secondary anti-Ig (FITC conjugates of mouse anti-rat IgG or goat anti-rabbit $F(ab')_2$, both from Jackson ImmunoResearch); (c) rat or rabbit IgG at 10 μg/ml to quench; and (d) the PE- or biotin-labeled antibody. The latter reagents were purchased from PharMingen (La Jolla, CA), and were: biotin conjugates directed to class II MHC (clone AMS-32.1), B220/CD45RB (clone RA3-6B2) and Thy-1.2/CD90 (clone 53-2.1) antigens; or PE conjugates directed to Mac-1/CD11b (clone M1/70) and Gr-1 granulocyte (clone RB6-8C5) antigens. At least 10,000 cells per sample were examined in a FACScan cytofluorograph (Becton Dickinson Immunocytometry Systems, Mountainview CA).

Immunoblotting-- This was performed with monoclonal and polyclonal reagents, as described (in Example 1, *supra*).

Immunocytochemistry-- Cytospins were fixed in neat acetone for 10 min at room temperature, air-dried, and stained with antibodies exactly as described for cytofluorography. The same secondary reagents were used, except that peroxidase conjugates instead of FITC conjugates were employed. Staining was visualized with diaminobenzidine (Stable DAB, Research Genetics, Huntsville, AL).

Functional Studies-- Monoclonal (10 and 1 μg/ml) and polyclonal (30 and 10 μg/ml) antibodies were applied at doses that were close to or above saturation, continuously, to a one-way allogeneic mixed leukocyte reaction (MLR), wherein 3 x 10^5 nylon wool-passed lymph node T cells were stimulated by graded doses of allogeneic irradiated or mitomycin-treated DCs (Inaba and Steinman, 1984, J. Exp. Med. 160:1717; Inaba et al., 1987, J. Exp. Med. 166:182). Syngeneic MLRs were run in tandem. The positive control for MLR inhibition utilized a reagent that

interferes with the B7 costimulation system (GL-1 rat mAb to B7-2) (Hathcock et al., 1993, Science 262:905). For *in vivo* experiments, we used the local graft-versus-host (GVH) reaction (Atkins and Ford, 1975, J. Exp. Med. 141:664), wherein parental lymph node cells injected into the hind foot pad induce a GVH in the draining popliteal node of F1 mice, presumably upon encountering F1 dendritic cells in the lymph node cortex. Lymph node weights of control (PBS-injected) and GVH nodes were measured at day 7, using 5 mice per group. The protocol was to inject 200 μg of anti-DEC-205 or control IgG into the foot pad at time 0, and to repeat the Ig injection 8 hours later8 hours later, adding either 10^7 parental lymph node cells or a corresponding volume of PBS to the injected solution.

Results

Expression of DEC-205 by epidermal dendritic cells. Three forms of antibody to DEC-205 (monoclonal NLDC-145, polyclonal anti-DEC-205 IgG, and polyclonal anti-DEC-205 $F(ab')_2$ fragments) were applied to cultured epidermal cells. Prior data had shown strong staining of Langerhans cells by NLDC-145. Since the NLDC-145 epitope is trypsin-sensitive (below), and since trypsin is used initially to prepare the sheets of epidermis from which Langerhans cells are released, we concentrated on epidermal cells that had been cultured overnight, to allow time for the protein to be repleted on cell surfaces. The culture period also provides time for most keratinocytes to adhere to the plastic surface, and for the nonadherent Langerhans cells to acquire a low buoyant density (Crowley et al. *supra*; Schuler and Steinman, *supra*). As a result, preparations with 20-50% dendritic cells can be obtained by studying nonadherent, overnight cultures of epidermal cells, especially following flotation on columns of dense BSA.

Epidermal cells were stained with a phycoerythrin (PE)-tagged mAb to class II MHC proteins, to distinguish Langerhans cells from keratinocytes, and were counterstained with hybridoma supernatants of NLDC-145 and mAbs to other leukocyte lineages (Figure 11, A-D). The specificity of NLDC-145 for dendritic cells (Figure 11A) was demonstrated by the fact that isotype-matched IgG2a mAbs

to macrophages (SER-4 anti-sialoadhesin (Coocker and Gordon, 1989, J. Exp. Med. 169:1333)), B cells (RA3-6B2 anti-B220 (Hoffman and Weissman, 1981, Nature 289:681)), and T cells (53-6.72 anti-CD8; (Ledbetter and Herzenberg, 1979, Immunol. Rev. 47:63)) were non-reactive with NLDC-145 (+) cells (Figure 11, panels B-D). When the same suspensions were counterstained with graded doses of either purified NLDC-145 IgG (rat IgG2aκ) or polyclonal, nonimmune rat IgG2a as a nonreactive control, staining of Langerhans cells by NLDC-145 reached a plateau at 2 μg/ml (Figure 11, compare the staining of the arrowed MHC-II (+) dendritic cells in panels E-G with H).

The anti-DEC-205 rabbit polyclonal was also applied, both as $F(ab')_2$ fragments and as intact IgG, and was compared with nonimmune $F(ab')_2$ and IgG over a broad range of doses (0.3-100 μg/ml). The rabbit reagents did react with the class II MHC-negative keratinocytes, but this binding was entirely nonspecific, since the staining was comparable with immune and nonimmune reagents (Figure 11, compare panels I-L with M-P, and Q-T with U-X). The staining of MHC-II (+) Langerhans cells, however, was strong, specific, and comparable using anti-DEC-205 as either intact IgG or $F(ab')_2$ fragments, with apparent saturation at 30 μg/ml (Figure 11, panels I-L for $F(ab')_2$ fragments, panels Q-T for intact IgG).

The second rabbit polyclonal antibody, raised to a synthetic peptide spanning the first 19 residues of DEC-205, failed to stain Langerhans cells, instead giving a pattern like that of nonimmune IgG (not shown).

The trypsin sensitivity of DEC-205 epitopes was examined. Partially-enriched cultured Langerhans cells were either not treated or were exposed to trypsin (0.25% in PBS for 30 min on ice). Staining by both monoclonal and polyclonal reagents was decreased by ten-fold (1 log of fluorescence intensity in Figure 12 compare A and B to C and D). The epitopes were reexpressed, to levels equal to those on untreated cells, during a subsequent overnight culture period (Figure 12, compare E and F with A and B).

Expression of DEC-205 by other dendritic cell populations. Spleen DCs were identified in low buoyant density splenocyte suspensions (See Example 1; and Surggard et al., *supra*) by their expression of the integrin CD11c (Figure 13A, C, E and G), as detected with the mAb N418 (Metlay et al., 1990, J. Exp. Med. 171:1753). Counterstaining with either NLDC-145 or with the anti-DEC-205 polyclonal revealed that, whether freshly isolated or cultured overnight, these cells expressed less DEC-205 on their surfaces than Langerhans cells (Figure 13C, D, G and H). Freshly-isolated spleen DCs comprised two phenotypic subsets, as previously described (Crowley et al., *supra*). Most (roughly 80%) expressed relatively low but detectable levels (≤1 log) of the antigen, while a smaller population stained moderately (ca. 1.5 logs: *arrows*, Figure 13C, D, G, and H). After overnight culture, DEC-205 expression by all of the CD11c (+) DCs had risen to the moderate (1.5 log) level, but never to the levels observed on epidermal dendritic cells (ca. 2 logs: *arrows*, Figure 13K, L, O and P) attempts to further upregulate expression of DEC-205 by culturing the cells in rGM-CSF, or in keratinocyte-conditioned medium containing GM-CSF, did not yield an increase beyond that induced by culture alone (not shown).

In contrast, when dendritic cells were generated from bone marrow progenitors with GM-CSF (Inaba et al., 1972, *supra*), their expression of DEC-205 was uniformly high, comparable to the levels on Langerhans cells (not shown). Interestingly, the actively proliferating populations at day 6 of the marrow cultures contained relatively few cells that expressed DEC-205 (Figure 14), but expression had increased to uniformly high levels by day 8.

Expression by resident and elicited peritoneal cells. Resident peritoneal cells (consisting of about 30% macrophages and 70% B cells) were compared to inflammatory peritoneal cells in exudates elicited with concanavalin A (Con A), thioglycollate (TGC), or live *M. bovis* Bacille Calmette-Guérin (BCG) organisms (Figure 15). The data are shown for polyclonal nonimmune and immune $F(ab')_2$ fragments. Similar staining was obtained with the NLDC-145 monoclonal (not

shown). However, nonimmune and immune intact rabbit IgG gave strong background staining on peritoneal macrophages, presumably because of binding to Fc receptors (not shown). Resident, Mac-1 (+) peritoneal macrophages did not express surface DEC-205, but peritoneal B cells expressed measurable levels (roughly 1 log above background: Figure 15 E-H *arrows*). Macrophages in Con A- and BCG-elicted exudates again showed little or no staining with anti-DEC-205 (Figure 15 E-H, *arrowheads*), even though these macrophages were all strongly class II MHC-positive (not shown). The Con A and BCG exudates contained significant numbers of T cells, but these did not stain with anti-DEC-205 (anti-Thy-1 double label not shown). In contrast to the other peritoneal populations tested, TGC-elicited macrophages did express DEC-205, albeit at low levels (0.5-1 log above background). In each of the resident and elicited populations, B cells stained comparably.

Expression of DEC-205 by resident leukocytes in multiple tissues, particularly B cells. Given the surprising finding that resident peritoneal B cells expressed DEC-205, we explored the distribution of the antigen further by examining cell suspensions from spleen, bone marrow, peripheral blood, lymph node, and thymus for co-expression of DEC-205 with several leukocyte markers. Results with the first 3 organs are illustrated here (Figure 16). The results with spleen and lymph node were identical (not shown). Thymocytes stained only marginally (<0.5 log) above background (not shown).

In spleen suspensions, B cells (B220 and MHC-II (+); Gr-1, Mac-1 and Thy-1 (-): Figure 16 F-J, *arrows*) again expressed DEC-205, staining approximately one log above background. T cells (Thy-1 (+)) and granulocytes (Gr-1 (+), Mac-1 (+)) also stained, but less strongly than B cells.

In bone marrow (Figure 16, K-T), granulocytes stained almost a log above background, whereas marrow B cells displayed heterogeneous levels of DEC-205. Most B220 (+) cells in the marrow stained weakly or not at all, while a subset of

cells with higher levels of B220 (*arrow*, Figure 16, P-T, B220 double label) co-expressed DEC-205 at levels comparable to those on peripheral B cells. These $B220^{bright}$,DEC-205 (+) cells also expressed surface IgM, identifying them as mature B cells (not shown). A small subset of Mac-1 (+), B220 (-) cells -- presumably monocytes -- did not express DEC-205.

In peripheral blood (Figure 16 U-δ,), B cells (*arrows*) stained comparably to B cells in lymphoid tissues, while granulocytes and T cells showed weaker but measurable staining.

To gain knowledge of the relative amounts of DEC-205 expressed by different cell types (Figure 17), graded doses of whole-cell NP-40 lysates from bone marrow dendritic cells, splenocytes (about 65% B cells) and peritoneal cells (about 70% B cells and 30% macrophages) were immunoblotted. The signal from 10,000 BMDCs was approximately twice as strong as the signal from 100,000 splenocytes, corresponding to at least ten times more DEC-205 per cell in BMDCs than splenic B cells. The signal from 100,000 peritoneal cells was weaker still, 5-10 times fainter than the signal from 10,000 DCs, corresponding to about 50 times less DEC-205 in peritoneal B cells than BMDCs.

To examine the effects of B cell activators on surface levels of DEC-205, B cells were cultured for up to 2 days in the presence of LPS, CD40 ligand, and the combination of anti-IgM and IL-4. None induced a significant increase, but the latter combination induced a modest (2-fold) decrease in surface levels of DEC-205, as detected with both monoclonal and polyclonal reagents (not shown).

Mouse B cell viability is greatly improved during stimulation in culture, allowing us to ask whether the DEC-205 epitopes on B cells were actively synthesized by the cells, rather than adsorbed on their surfaces from an extracellular source. On B cells, as on Langerhans cells, trypsin treatment eliminated most of the staining by

NLDC-145 and anti-DEC-205, but the epitopes were resynthesized during overnight culture (Figure 12 G-L).

Attempts to block dendritic cell function with antibodies to DEC-205. When one-way mixed leukocyte reactions were performed in the continuous presence of either 10 μg/ml of NLDC-145, or 30 μg/ml polyclonal anti-DEC-205, no inhibition of allogeneic T cell proliferation was observed (Figure 18). The rat IgG2a monoclonal GL-1, directed against the costimulator protein B7-2 (Hathcock et al., *supra*; Freeman et al., 1993, J. Exp. Med. 178:2185; Inaba et al., 1994, J. Exp. Med. 180:1849), was utilized as a positive blocking control. GL-1 inhibited proliferation in this system, but did not abolish it, as expected: blockade of multiple costimulators is required to completely ablate proliferation in an allogeneic MLR (Young et al., 1992, J. Clin. Invest. 90:229).

To ascertain whether DEC-205 might play a role in the homing of T cells to the T-dependent areas of lymphoid tissue, where dendritic cells express high levels of DEC-205 (Kraal et al., *supra*; Example 4, *supra*), the local graft-versus-host (GVH) reaction was studied (Atkins and Ford, *supra*). This response is produced in the draining popliteal lymph node of F1 mice when parental-strain lymph node cells are injected into the hind foot pad. The T cells migrate to the draining node, where they encounter allogeneic MHC proteins, presumably initially on dendritic cells and within the cortical T cell areas. A substantial dose of nonimmune or anti-DEC-205 $F(ab')_2$ fragments (200 μg) was injected into the footpad at 0 hours. Eight hours later, a second dose of antibody along with 10 million lymph node cells (ca. 70% T cells) was injected. The T cells induced a strong GVH reaction: the draining lymph nodes swelled to 5-6 times their normal size within 7 days. Polyclonal anti-DEC-205 was unable to inhibit this primary T cell response *in vivo* (Table 3).

Table 3: Failure of polyclonal anti-DEC-205 to inhibit l cal GVH *in vivo*

F1 footpad	Mouse number	Weight of popliteal lymph node (g) No cells injected	10^7 parental lymph node cells injected
Nonimmune $F(ab')_2$	1	2.63	13.27
	2	2.36	13.83
	3	2.89	12.01
	4	2.06	12.80
	5	2.14	13.93
Mean difference (GVH) = 10.75 ± 1.03 g			
Anti-DEC-205 $F(ab')_2$	1	2.88	14.91
	2	2.08	12.01
	3	2.22	13.19
	4	1.95	11.31
	5	2.38	13.33
Mean difference (GVH) = 10.65 ± 1.03 g			

Discussion

Prior studies failed to detect the DEC-205 antigen on most types of leukocytes except for dendritic cells (Kraal et al., *supra*; Crowley et al., *supra*). This Example demonstrates that other leukocytes, especially B cells, can express DEC-205, although at much lower levels. Example 4 reports low-level expression of DEC-205 by B cells in tissue sections. Polyclonal rabbit antibodies raised against the N-terminal peptide of DEC-205 and the intact protein described in Example 1 were used. The anti-peptide reagent failed to stain live cells, suggesting that under native conditions, the amino terminus of DEC-205 may be involved in a higher-order protein structure that alters its conformation relative to the 19-residue synthetic immunogen, or sterically hinders access by an antibody. The failure of the anti-peptide polyclonal to bind cells cannot be explained by covalent modification of the N-terminal serine of DEC-205 in tissues, since this residue was susceptible to Edman degradation in protein isolated from thymi. In contrast, the

polyclonal raised to intact DEC-205 stained cells with patterns of reactivity that mimicked NLDC-145 closely.

Differences in DEC-205 expression among different classes of leukocytes were observed. Other than dendritic cells, B cells expressed the most DEC-205, although their levels were 10 to 50 times lower than those on BMDCs (Figure 17). The DEC-205 detected on B cell surfaces was actively synthesized by the cells themselves, and unlikely to be adsorbed to their surfaces from an extracellular source, since after trypsinization, DEC-205 epitopes were regenerated in culture. Expression of DEC-205 appears to be coordinated with the developmental transition from pre-B cell to surface IgM (+) B cell in the marrow. However, peripheral B cell stimulation with a variety of mitogens (LPS, CD40 ligand, anti-IgM plus IL-4) was not accompanied by a significant increase in surface expression of DEC-205. Granulocytes expressed DEC-205, with higher levels on granulocytes in bone marrow than in blood. Thymocytes and mature T cells from spleen and lymph node expressed very low but detectable levels of DEC-205, whereas T cells in blood and peritoneal fluid did not express detecable levels. It is possible that the DEC-205 detected on granulocytes and T cells was adsorbed from surrounding stromal cells that are rich in DEC-205, such as bone marrow stroma, thymic epithelium, and the dendritic cells in the T cell areas of peripheral lymphoid tissues. Most macrophage populations lack DEC-205, although thioglycollate-elicited cells are weakly positive, as previously described (Wiffels et al., 1991, Immunobiol. 184:83).

Although other leukocyte lineages express DEC-205, it is evident that dendritic cells express some 10-50 times more of the antigen, as assessed by immunoblotting. DEC-205 expression is regulated on dendritic cells in some way. Freshly isolated splenic DCs have relatively little DEC-205, and the levels increase only modestly in culture. The dendritic cells that express high levels of DEC-205 are those in skin, in the T cell regions of peripheral lymphoid organs, and dendritic

cells that are grown from proliferating bone marrow precursors in the presence of high-dose GM-CSF.

To look for a contribution of DEC-205 to immune responses in tissue culture systems, we attempted to inhibit the primary allogeneic MLR. It was hypothesized that DEC-205 might be important in the capacity of dendritic cells to interact with helper T cells. However, either monoclonal nor polyclonal antibodies were able to block this T cell response with either monoclonal or polyclonal antibodies. We also attempted to inhibit an *in vivo* response, the local GVH reaction, in which parental T cells injected into nonlymphoid tissue migrate to the lymph node that drains the injection site and initiate an alloreactive response against F1 dendritic cells and B cells. Again, antibodies to DEC-205 had no effect. These negative results could have trivial explanations, such as clearance of the antibody, or compensatory resynthesis of new DEC-205 protein. Given the large molecular mass of DEC-205, it is possible that both monoclonal and polyclonal antibodies bind epitopes on the native protein that do not interfere with its functions in the responses studied. Alternatively, the assay systems may be those in which DEC-205 plays no critical role. DEC-205's putative function in immune responses appears to involve earlier events than those studied here, such as the acquisition of antigens by accessory cells, or selection events in lymphocyte development. Further inquiries into the function of DEC-205 are enabled by the molecular cloning of the antigen described in Example 2, *supra*.

EXAMPLE 4: EXPRESSION OF THE DEC-205 PROTEIN *IN SITU* IN LYMPHOID AND NONLYMPHOID TISSUES

In this Example, the monoclonal and polyclonal antibodies to DEC-205 were used to reassess the tissue distribution of DEC-205 by immunohistochemical staining of frozen sections from a variety of organs, and by multiple-organ immunoblotting.

In an effort to better define the tissue distribution of DEC-205, we have examined a variety of tissues histologically and on immunoblots, using both monoclonal and

polyclonal antibodies, along with secondary anti-Ig reagents with greater sensitivity than those used in prior studies (Kraal et al., 1986, J. Exp. Med. 163:981; Crowley et al., 1989, Cell. Immunol. 118:108; Vremec et al., 1992, J. Exp. Med. 176:47; Austyn et al., 1994, J. Immunol. 152:2401; Lu et al., 1994, J. Exp. Med. 179:1823; Breel et al., 1988, Immunology 63:657). Abundant expression of DEC-205 was confirmed histologically on thymic and intestinal epithelia and on dendritic cells in the T cell areas of peripheral lymphoid organs. In addition, DEC-205 was visualized in several other locations: B lymphocytes within B cell follicles, the stroma of the bone marrow, the epithelia of pulmonary airways, and the capillaries of the brain. Immunoblotting confirmed the presence of substantial levels of DEC-205 protein in lysates prepared from lymphoid tissues and from lung, marrow and intestine. Thus, while DEC-205 is expressed at high levels by dendritic cells, it is also expressed by a number of other cell types *in situ*.

Material and Methods

Mice -- Adult (6-12 week old) female mice of three strains were studied: inbred C57BL/6 x DBA/2 (Trudeau Institute, Saranac Lake, NY) and BALB/C, and outbred CD-1 Swiss-Webster (the latter 2 strains from Taconic Farms, Germantown, NY).

Immunohistology -- Immediately after organs were removed, they were frozen at -20°C in O.C.T. tissue embedding medium (Miles, Elkhart, IN), and stored at -20°C. Tissue sections, usually 10 μm thick, were cut on a Minotome cryostat (IEC division of Damon, Needham Heights, MA) and applied to 10-well slides (Carlson Scientific, Peotone, IL). The sections were fixed in neat acetone for 10 min at room temperature, and air-dried. Subsequent steps were performed in a humid chamber. Sections were rehydrated in a drop (30-50 μL) of PBS, then primary antibody was applied. Hybridoma supernatants were used either undiluted or diluted 1:5 in PBS + 1% BSA, depending on their titer. Purified IgGs, ascites fluids and antisera were diluted in the same medium to optimized doses determined by titration, usually 1-10 μg/ml for purified protein and 1:3000-1:1000 for ascites

and hyperimmune sera. The primary antibodies were the NLDC-145 mAb, applied either as a hybridoma culture supernatant or as purified IgG (protein G eluate), or rabbit polyclonal anti-DEC-205, (Example 1 *supra*), applied either as intact IgG (protein A eluate) or as $F(ab')_2$ fragments. Positive controls included rat mAbs to other subsets of leukocytes (RA3-6B2 anti-B220/CD45RB (TIB 146, ATCC, Rockville , MD), SER-4 anti-macrophage, and 53-6.72 anti-CD8 (TIB 105, ATCC)) and MHC class II proteins (M5/114 (TIB 120, ATCC)), as well as a rabbit polyclonal to Igβ, one of the signaling chains that associates with B cell surface Ig (Sanchez et al., 1993, J. Exp. Med. 178:1049). Negative controls were polyclonal rat IgG2a (Zymed, South San Francisco, CA) and rabbit IgG (Jackson ImmunoResearch, West Grove, PA), either intact or as $F(ab')_2$ fragments. Primary antibodies were left in contact with the sections for 45 min at room temperature, then the sections were washed 5 times with PBS, never allowing the sections to stand dry for more than a few seconds. Anti-Ig secondary antibodies were added next, usually donkey $F(ab')_2$ - horseradish peroxidase conjugates (Jackson ImmunoResearch), diluted 1:300 in PBS + 1% BSA. After a 45 minute incubation, sections were washed 5 times with PBS. The chromogenic substrate was a ready-to-use formulation of diaminobenzidine containing H_2O_2 (Stable DAB, Research Genetics, Huntsville, AL). Sections were washed 5 times in PBS, and usually were counterstained with Gill's Hematoxylin #1 (Fisher, Fair Lawn, NJ). Coverslips were attached using Permount histological mounting medium (Fisher).

Extraction and immunoblotting of protein from multiple organs -- Organs were placed in 10 volumes of a monophasic solution of phenol and guanidinium isothiocyanate (TRIzol, Gibco-BRL, Gaithersburg, MD) (Chomczynski, 1993, BioTechniques 15:532). Organs were homogenized for 15-30 s (Polytron, Brinkmann, Westbury, NY). RNA and DNA were removed by $CHCl_3$ extraction and ethanol precipitation. Proteins were precipitated from the phenol-ethanol supernatants with isopropanol (150% of the original TRIzol volume), and were redissolved in 1% SDS (30% of the original TRIzol volume, 50°C, 1 h). Extracts were clarified (3000 x g, 10 min, room temperature), and total protein levels were

measured (BCA assay, Pierce, Rockford, IL). Immunoblotting was performed as described (Chomczynski, *supra*), normalizing protein loads to 50 μg per lane. Filters were stained with either 10 μg/ml of NLDC-145 IgG or mAb 1D4B (anti-LAMP-1) hybridoma supernatant, diluted 1:1.

Results

In every organ studied, similar results were obtained with the NLDC-145 monoclonal antibody and with the rabbit polyclonal antibody raised to purified DEC-205 protein. This will be illustrated in several lymphoid and nonlymphoid tissues. In many instances, DEC-205 was found to be expressed in sites that had not been described in prior work.

Thymus. Staining patterns in this organ were identical to those in the original description of the NLDC-145 mAb (Kraal et al., *supra*). Very strong peroxidase immunolabeling was observed on thymic cortical epithelium, while weaker staining was noted on scattered dendritic profiles in the medulla (M, Figure 20, panels *a-c* and *g*).

Lymph node. Strong DEC-205 expression was apparent on dendritic profiles throughout the T cell regions of the cortex (T, Figure 20 *d-f*). At higher power, there was a punctate character to the staining in the T cell areas of the deep cortex (Figure 20 *h*). The punctate pattern could represent the presence of DEC-205 in intracellular granules of dendritic cells and/or DEC-205 on the surfaces of many fine processes. No staining was evident in the medulla (M, Figure 20 *d-f*). Weak staining for DEC-205 was evident on B cells in the follicles (B, Figure 20 *d-f*). This staining was obscured when hematoxylin was used to counterstain nuclei (Figure 20 *d-f*), but was clearly visible when hematoxylin was omitted (Figure 20 *i*).

Spleen. Strong staining for DEC-205 was evident in the T cell areas, *i.e.*, the periarterial sheaths (Figure 21: the central artery of the T area is arrowed in each

micrograph). The extent of staining for DEC-205 in the T areas paralleled that seen with anti-MHC class II (Fig. 21, compare *b* with *c*, and *d* and *e* with *f*), but the DEC-205 stain was again punctate in nature (Figure 21 *d-e*), as it was in the T cell areas of lymph nodes (above). As in lymph nodes (above), the B cell follicles (B, Figure 21 *a-c*) stained for DEC-205, although this staining was too weak to be evident in a black and white micrograph of a specimen counterstained with hematoxylin (Figure 21, compare the strong B cell staining with anti-Igβ in panel *a* and anti-MHC-II in panel *c* with the anti-DEC-205 staining in panel *b*). Anti-DEC-205 did not stain the marginal zone (see *arrowheads* to the marginal sinus, which stands out in panels *a* and *c* because of the strong B cell staining).

Brain. Both NLDC-145 and polyclonal anti-DEC-205 reagents produced linear staining along capillaries (*arrows*, Figure 22 *a-c*) and small arteries (*arrow*, Figure 22 *d*) in the cerebrum and cerebellum. Staining of capillaries was not observed in any other organ studied.

Lung. Many strongly stained DEC-205 profiles were scattered about the lung parenchyma (Figure 22 *e*, *g*, *h*). We have not yet determined if these profiles represent dendritic cells, macrophages or both. Some strongly stained cells within the airways, presumably alveolar macrophages, were evident (*, Figure 22 *h*). DEC-205 was present in the epithelium of all the small airways (*arrows*, Figure 22 *e*, *h*). In contrast, anti-MHC class II did not stain the airway epithelium, but did stain cells surrounding the airways (Figure 22 *f*, *arrowheads*).

Bone marrow. When bone marrow was extruded from the femur as an intact plug and sectioned, a lacy pattern of DEC-205 stain was evident throughout the plug, presumably on marrow stromal cells (*arrows*, Figure 22 *i*). Most of the dark staining of round cells represented background staining of eosinophils, which express endogenous peroxidases. It was evident in the absence of any antibody (not shown).

Upper gastrointestinal tract. The oral epithelium of the tongue served as an example of a stratified squamous epithelium. Some DEC-205 positive profiles, presumably Langerhans cells, were found suprabasally (*arrows*, Figure 22 *j*). Anti-MHC class II antibodies stained these intraepithelial dendritic cells more frequently and/or more intensely, and in addition stained many subepithelial profiles in the upper dermis (not shown).

Lower gastrointestinal tract. Strong staining was observed on the columnar epithelia of the small and large bowel. The staining was much greater on apical villi than on crypt epithelium (Figure 22, *k-l*). In the best sections, staining was also stronger along the basal surfaces than the apices of individual epithelial cells. Many cells within the lamina propria of the villi stained darkly, but this staining was again due to endogenous peroxidase within eosinophils.

Liver. No staining for DEC-205 was apparent, except for rare profiles in the portal triads (not shown).

Heart. No staining for DEC-205 was apparent (not shown).

Kidney. No strong expression of DEC-205 was noted, although some very weak DEC-205 staining was observed on scattered cortical tubules (not shown).

Distribution of DEC-205 by multiple-organ immunoblotting. To examine the tissue distribution of the DEC-205 protein itself, TRIzol protein extracts of several different organs (Methods) were immunoblotted with both monoclonal NLDC-145 IgG (Figure 23A) and polyclonal anti-DEC-205 IgG (not shown). The quantities of organ lysate protein applied to each lane were normalized to one another in terms of protein load (50 μg per lane). Staining for the lysosomal membrane marker LAMP-1 (Chen et al., 1985, Arch. Biochem. Biophys. 239:574) revealed that comparable numbers of lysosomes were represented in each lane (Figure 4B). Among lymphoid tissues, the signal for DEC-205 was greatest in the thymus, and

was stronger in bone marrow and lymph nodes than in the spleen. These findings corresponded closely to the histologic staining levels described above. In nonlymphoid tissues, strong signals comparable to thymus were evident in lung and intestine (Figure 4A). Liver, kidney and brain extracts contained only trace levels of DEC-205.

Discussion

Using an improved donkey $F(ab')_2$ secondary anti-rat Ig reagent to increase the sensitivity of antigen detection, we have shown that the NLDC-145 monoclonal antibody reacts with many more tissues than previously apparent. In particular, clear staining was observed on brain capillaries, bone marrow stroma, the epithelia of intestinal villi and pulmonary airways, and B cells in the follicles of all peripheral lymphoid tissues. These newly-recognized depots of DEC-205 antigen did not stain as strongly as tissues that were originally noted to express the DEC-205 protein, *i.e.*, the cortical epithelium of the thymus, and the dendritic cells in the T cell areas of peripheral lymphoid organs.

The tissue distribution of the DEC-205 protein was confirmed with a polyclonal antibody raised to purified DEC-205. Both $F(ab')_2$ and intact IgG forms of the rabbit antibody gave patterns of staining that were similar to those obtained with monoclonal NLDC-145. The tissue distribution was also monitored by immunoblotting with both monoclonal and polyclonal reagents, and the relative levels of expression in multiple organs corresponded to the relative intensities of immunohistochemical staining seen on tissue sections. An exception is that trace amounts of DEC-205 protein were evident in extracts from organs like liver, heart, and kidney where it was difficult to appreciate discrete DEC-205 positive cells. This suggests that very small amounts of the protein may be present in many cell types, but at levels too low to permit histologic visualization.

The present invention is not to be limited in scope by the specific embodiments described herein since such embodiments are intended as but single illustrations of one aspect of the invention and any embodiments which are functionally equivalent are within the scope of this invention. It should be further understood that all molecular weight and nucleotide base pair sizes given for nucleotides are approximate and are used for the purpose of description. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference herein in their entireties.